PCT/EP2004/051022

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Patentanmeldung Nr. Patent application No. Demande de brevet n°

03076719.8

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Anmeldung Nr:
Application no.: 03076719.8
Demande no:

Anmeldetag:
Date of filing: 03.06.03
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
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Plants having modified growth characteristics and a method for making the same

In Anspruch genommene Prioriät(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N15/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT RO SE SI SK TR LI

097-OsNHX1-PROV

Plants having modified growth characteristics and a method for making the same

The present invention concerns a method for modifying plant growth characteristics. More specifically, the present invention concerns a method for modifying plant growth characteristics by modulating expression of a nucleic acid encoding a NHX protein and/or by modulating activity and/or levels of a NHX protein in a plant. The present invention also concerns plants having modulated expression of a nucleic acid encoding a NHX protein and/or modulated activity and/or levels of a NHX protein, which plants have modified growth characteristics relative to corresponding wild type plants. More particularly said transgenic plants, when grown in non-saltstress conditions, have improved growth characteristics compared to the wild-type plants and outperform wild-type plants also grown on the same non-saltstress conditions.

Given the ever-increasing world population, and the dwindling supply of arable land available for agriculture, it remains a major goal of agricultural research to improve the efficiency of agriculture and to increase the diversity of plants in horticulture. Conventional means for crop and horticultural improvements utilise selective breeding techniques to identify plants having desirable characteristics. However, such selective breeding techniques have several drawbacks, namely that these techniques are typically labour intensive and result in plants that often contain heterogeneous genetic components that may not always result in the desirable trait being passed on from parent plants. Advances in molecular biology have allowed mankind to manipulate the germplasm of animals and plants. Genetic engineering of plants entails the isolation and manipulation of genetic material (typically in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant. Such technology has led to the development of plants having various improved economic, agronomic or horticultural traits. Traits of particular economic interest are growth characteristics such as high yield.

The NHX protein is a sodium antiporter and as an active sodium pump the NHX protein is involved in extruding Na+ ions from the cytoplasm into the vacuole of a cell. This is one of the mechanisms of a plant to protect the cells against high salinity in the soil and the water. NHX genes were isolated from a number of plant species, such as from *Arabidopsis* (Gaxiola et al. 1999. PNAS 96, 1480-1485), from rice (OsNHX, (Fukuda et al. Molecular cloning and expression of the Na+/H+ exchanger gene in *Oryza sativa*. Biochim Biophys Acta. 1999 Jul 7;1446(1-2):149-55) and from *Atriplex* (AgNHX, JP2000157287).

Transgenic plants overexpressing the *Arabidopsis* gene AtNHX have been shown to have increased tolerance to high salinity (200 to 400 mM NaCL) in the growth media. Examples of such salt-tolerant *Arabidopsis* plants, tomato's and Brassica have been described. (Apse et al., Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis. Science. 1999 Aug 20;285(5431):1256-8; Apse MP, Blumwald E. Engineering salt tolerance in plants. Curr Opin Biotechnol. 2002 Apr;13(2):146-50; Zhang and Blumwald Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat Biotechnol. 2001 Aug;19(8):765-8; Zhang et al. Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc Natl Acad Sci U S A. 2001 Oct 23;98(22):12832-6). The transgenic *Brassica napus* plants overexpressing AtNHX, were able to grow, flower, and produce seeds in the presence of 200 mM sodium chloride. Although the transgenic plants grown in high salinity accumulated sodium up to 6% of their dry weight, growth of the these plants was only marginally affected by the high salt concentration. Moreover, seed yields and the seed oil quality were not affected by the high salinity of the soil.

Futher, also salt tolerant monocots were generated via the transformation of an NHX gene. Ohta et al. (FEBS Lett. 2002 Dec 18;532(3):279-82) engineered a salt-sensitive rice cultivar (*Oryza sativa* cv. Kinuhikari) to express a vacuolar-type Na+/H+ antiporter gene from the halophytic plant, *Atriplex gmelini* (AgNHX). The activity of the vacuolar-type Na+/H+ antiporter in the transgenic rice plants was eight-fold higher than that in wild-type rice plants. Salt tolerance assays followed by non-saltstress treatments showed that the transgenic plants overexpressing AgNHX could survive under conditions of 300 mM NaCl for 3 days while the wild-type rice plants could not. This result indicates that overexpression of the Na+/H+ antiporter gene in rice plants significantly improves their salt tolerance. After salt-stress treatments, the surviving transgenic rice plants were transferred to soil conditions without saltstress and grown in the green house. Although the number of tillers was reduced compared to untreated transgenic rice plants, the transgenic rice plants grew until the flowering stage and set seeds after 3.5 months, demonstrating that the salt shock did not completely damage the fertility of the transgenic rice plants

All these transgenic plants showed better survival capacity when grown on high salinity media and show "wild-type phenotypes" on the green biomass level and on the level of flowering and seed-production, while the non-transgenic plants were suffering from salt toxicity. Except in tomato, where the fruits of the transgenic plants were smaller than the fruits from the wild-type non-saltstressed plants.

In summary, several reports have established a role of NHX genes in salt tolerance.

Unexpectedly, it has now been demonstrated in the present invention that overexpression of a OsNHX gene leads to an increase in yield in a rice plant grown under normal, non-saltstressed, growing conditions. The transformed rice plants of the present invention outperform the normal wild-type plants with several improved growth and yield parameters.

Therefore, in the present invention, it has now been shown that NHX genes can be used to improve growth characteristics of a transformed plant, when compared to a wild-type plant. It is demonstrated for the first time that transgenic plants, transformed with an NHX gene, can outperform the growth characteristics of a wild-type plant, when both the transgenic and the wild-type plants are grown under normal or optimal, meaning non-saltstress, growth conditions.

Therefore, it is shown that growth characteristics under non-saltstress conditions may be improved via genetic manipulation of one gene, one trait, in a plant and that by modulating expression of a nucleic acid encoding a NHX protein in a plant gives rise to plants having modified growth characteristics, preferably improved growth characteristics, when compared to the wild-type plants. Therefore according to a first embodiment of the present invention there is provided a method for modifying the growth characteristics of a plant grown under non-saltstress conditions, comprising modulating expression in a plant of a nucleic acid encoding a NHX protein and/or modulating activity and/or levels in a plant of a NHX protein.

Modulating (enhancing or decreasing) expression of a nucleic acid encoding a NHX protein or modulation of the activity and/or levels of the NHX protein itself encompasses altered expression of a gene and/or altered activity and/or levels of a gene product, namely a polypeptide, in specific cells or tissues. Modulating expression of a gene and/or modulating activity and/or levels of a gene product may be effected, for example by chemical means and/or recombinant means. Modulating expression of a gene and/or modulating activity and/or levels of a gene product may be effected directly through the modulation of expression of a NHX encoding gene and/or directly through the modulation of the activity and/or levels of a NHX protein. Additionally or alternatively, the modulation of expression as mentioned above is effected in an indirect way, for example may be effected as a result of decreased or increased levels and or activity of factors that control the expression of a NHX gene or that influence the activity and/or levels of the NHX protein.

Advantageously, modulation of expression of a nucleic acid encoding a NHX protein and/or modulation of activity and/or levels of the NHX protein itself may be effected by chemical means, i.e. by exogenous application of one or more compounds or elements capable of modulating activity and/or levels of the NHX protein and/or capable of modulating expression

of an NHX gene The exogenous application may comprise contacting or administering cells, tissues, organs or organisms with the gene product, preferably the NHX gene product, or a homologue, derivative or active fragment thereof and/or to antibodies recognizing or mimicking the gene product. Such antibodies may comprise "plantibodies", single chain antibodies, IgG antibodies and heavy chain camel antibodies, as well as fragments thereof.

A more indirect way for chemical modulation of expression of a nucleic acid encoding a NHX protein and/or chemical modulation of activity and/or levels of the NHX protein itself, may also be effected as a result of manipulation of factors that directly or indirectly activate or inactivate a NHX protein. Such factors may be upstream regulators involved in the signal transduction pathways leading to NHX activity. For osmotic stress signal-transduction involved MAP kinases and MYC/MYB transcription factors, while for ionic stress signal-transduction involved calcium-dependent kinases, for example the calcium-activated protein kinase complex SOS3-SOS2 etc...
Additionally or alternatively, contacting or administering cells, tissues, organs or organisms with an interacting protein or to an inhibitor or activator of the gene/gene product provides another exogenous means for modulation of expression of a nucleic acid encoding a NHX protein and/or for modulation of activity and/or level of the NHX protein itself. Such factors might cause the NHX protein to take on an inactive state and decrease the availability of (functional) NHX proteins in the cell. Similarly, factors promoting the NHX protein to an active state would in turn cause an increase in availability of NHX protein.

Therefore, according to one aspect of the present invention, there is provided a method for modifying the growth characteristics of a plant and/or a method for the production of plants or plant parts having modified growth characteristics, comprising exogenous application of one or more compounds or elements capable of modulating expression of a NHX gene and/or capable of modulating activity and/or levels of a NHX protein.
Examples of such compounds are proteins, as described above, or nucleic acids.

Therefore, additionally or alternatively, and according to a preferred embodiment of the present invention, modulation of expression of a nucleic acid encoding a NHX protein and/or modulation of activity and/or levels of the NHX protein itself may be effected by recombinant means. Such recombinant means may comprise a direct and/or indirect approach for modulation of expression of a nucleic acid and/or for modulation of the activity and/or levels of a protein.

For example, an indirect recombinant approach may comprise for example introducing, into a plant, a nucleic acid capable of modulating activity and/or levels of the protein in question (a NHX protein) and/or capable of modulating expression of the gene in question (a gene encoding a NHX protein). Examples of such nucleic acids to be introduced into a plant, are nucleic acids encoding transcription factors or activators or inhibitors that influence the expression of the NHX gene or that influence the activity and/or level of the NHX protein. Examples of such factors are upstream regulators involved in the signal transduction pathways leading to NHX activity. For osmotic stress signal-transduction involved MAP kinases and MYC/MYB transcription factors, while for ionic stress signal-transduction involved calcium-dependent kinases, for example the calcium-activated protein kinase complex SOS3-SOS2 etc...

The NHX gene or the NHX protein may be wild type, i.e. the native or endogenous nucleic acid or polypeptide. Alternatively, it may be a nucleic acid, which gene is introduced as a transgene. Also encompassed by an indirect approach for modulating activity and/or levels of a NHX protein and/or expression of a NHX gene is the provision of or the inhibition or stimulation of regulatory sequences that drive expression of the native gene encoding a NHX or the transgene encoding a NHX. Such regulatory sequences may be introduced into a plant. For example, the nucleic acid introduced into the plant is promoter, capable of driving the expression of an endogenous NHX gene.

Further, modulation of expression of a nucleic acid encoding a NHX may be effected by altering levels in a plant of a factor able to interact with NHX. Such factors may include ligands of the NHX. Therefore, the present invention provides a method to modify growth characteristics of a plant, comprising modifying expression of a gene coding for a protein, which is a natural ligand of a NHX. Furthermore, the NHX in its biological role in the cell, functions trough the targeting of several substrates. Therefore, the present invention also provides a method to modify growth characteristics of a plant relative to corresponding wild-type plants, comprising modifying expression of a gene coding for a protein, which is a natural target/substrate of a NHX. Preferably, the modification of expression results in an increase of the level of the target protein.

On the other hand, for modulating expression of a NHX gene or modulating the activity and/or levels of a NHX protein, a direct and most preferred approach comprises introducing into a plant a nucleic acid sequence encoding a NHX protein or a homologue, derivative or active fragment thereof. The nucleic acid may be introduced into a plant by, for example transformation.

A more preferred way comprises the introduction into a plant if a NHX gene as presented in SEQ ID NO 1, or a gene essentially similar to SEQ ID NO 1, or a gene encoding a protein essentially similar to SEQ ID NO 2. A most preferred way comprises the introduction into a plant of a NHX encoding gene in the sense direction, coupled to a tissue-specific promoter to improve growth characteristics of a plant grown under non-saltstress conditions, compared to a wild-type plant also grown under non-saltstress conditions.

Therefore according to one preferred aspect of the present invention, there is provided a method for modifying the growth characteristics of plants and a method for the production of a plant having such modified growth characteristics, comprising introducing, into a plant, a nucleic acid sequence capable of modulating expression of a gene encoding a NHX protein and/or capable of modulating the activity and/or levels of a NHX protein.

As mentioned above the nucleic acid to be used in the methods of the present invention can be wild type or native or endogenous. Alternatively, the nucleic acid may be derived from another species, which gene is introduced into the plant as a transgene, for example by transformation. The nucleic acid may thus be derived (either directly or indirectly (if subsequently modified)) from any source provided that the nucleic acid, when expressed in a plant, leads to modulated expression of a NHX nucleic acid/gene or modulated activity and/or levels of a NHX protein. The nucleic acid may be isolated from a microbial source, such as bacteria, yeast or fungi, or from a plant, algae or insect or animal (including human) source. This nucleic acid may be substantially modified from its native form in composition and/or genomic environment through deliberate human manipulation. The nucleic acid sequence is preferably a homologous nucleic acid sequence, for example a nucleic acid sequence obtained from another plant, whether from the same plant species or different. The nucleic acid sequence may be isolated from a monocotyledoneous species, preferably from the family Poaceae, further preferably from *Oryzaceae*. More preferably, the nucleic acid is as represented by SEQ ID NO: 1 or a portion thereof or a nucleic acid sequence capable of hybridising therewith or is a nucleic acid encoding an amino acid represented by SEQ ID NO: 2 or a homologue, derivative or active fragment thereof.

According to a preferred feature of the present invention, the modulation of expression of a NHX protein is the improvement of expression. Therefore, according to a preferred embodiment of the invention, enhanced or increased expression of a NHX protein encoding nucleic acid is envisaged. Methods for obtaining enhanced or increased expression of genes or gene products are well documented in the art and include, for example, overexpression driven by a (strong) promoter, the use of transcription enhancers or translation enhancers. The

term overexpression as used herein means any form of expression that is additional to the original wild-type expression level. In a preferred embodiment of the present invention the NHX gene is overexpressed in a plant via the introduction into a plant of a NHX gene in the sense orientation, to modify the growth characteristics of that plant. According, to a preferred embodiment, the present invention relates to a method to alter growth characteristics in a plant, comprising introducing, into a plant, a nucleic acid sequence in the sense orientation capable of modulating expression of a gene encoding a NHX and/or capable of modulating activity of a NHX protein.

Alternatively and/or additionally, increased expression of a NHX encoding gene or increase activities an/or levels of a NHX protein in a plant cells, is achieved by mutagenesis of the plant cell. For example these mutations can be responsible for the altered control of the NHX encoded gene, resulting in more expression of the gene. These mutations can also cause conformational changes of the protein, resulting in more activity and/or levels of the protein.

Mutations in the NHX gene may occur naturally, and may form the basis of the selection of plants showing higher yield. These allelic variants may have improved activity, for example via the improved affinity for ions. Therefore, according to another aspect of the invention, there is provided a method for the selection of pants having altered growth characteristics, which altered growth characteristics are based on altered activity of the NHX gene.

Modulating gene expression also encompasses altered transcript level of a gene. Altered transcript levels can be sufficient to induce certain phenotypic effects, for example via the mechanism of cosuppression. Here the overall effect of overexpression of a transgene is that there is less activity in the cell of the protein encoded by a native gene having homology to the introduced transgene. Cosuppression is accomplished by the addition of coding sequences or parts thereof in a sense orientation. Therefore, according to one aspect of the present invention, the growth characteristics of a plant may be modified by introducing into a plant an additional copy (in full or in part) of a NHX gene already present in a host plant. The additional gene may silence the endogenous gene, giving rise to a phenomenon known as co-suppression.

Techniques directly aimed at decreasing expression are also well documented in the art and include, for example, downregulation of expression by anti-sense techniques, RNAi techniques, small interference RNAs (siRNAs), microRNA (miRNA), the use of ribozymes, etc. Accordingly, downregulation of the nucleic acid sequence may also give rise to modified growth characteristics in a plant.

Downregulation of gene expression is also previously described in Atkins *et al.* 1994 (WO 94/00012), Lenee *et al.* 1995 (WO 95/03404), Lutziger *et al.* 2000 (WO 00/00619), Prinsen *et al.* 1997 (WO 97/3865) and Scott *et al.* 1997 (WO 97/38116). Further, gene silencing may be achieved by insertion mutagenesis (for example, T-DNA insertion or transposon insertion). Other gene silencing strategies are further described by, among others, Angell and Baulcombe 1998 (WO 98/36083), Lowe *et al.* 1989 (WO 98/53083), Lederer *et al.* 1999 (WO 99/15682) or Wang *et al.* 1999 (WO 99/53050). Expression of an endogenous NHX gene may also be reduced if there is a mutation on the endogenous gene.

Therefore according to a particular aspect of the invention, there is provided a method for modulating growth characteristics of plants, including the synthesis of antisense transcripts, complementary to the mRNA of NHX gene, or including the technique of co-suppression of a NHX gene, or including the use of RNAi technique, or based on RNA interference or siRNA's or micro RNA's or ribozymes or mutagenesis.

The expression "NHX protein" as used herein encompasses a protein essentially similar to SEQ ID NO 2. A gene encoding a NHX protein or a NHX gene (or NHX gene) encompasses a gene essentially similar to SEQ ID NO 1. The term "essentially similar to" SEQ ID NO 1 or 2 includes SEQ ID NO 1 or SEQ ID NO 2 itself and includes homologues, derivatives and functional fragments of SEQ ID NO 1 or 2. The term "essentially similar to" also includes at least a part or a portion of sequences SEQ ID NO 1 or SEQ ID NO 2.
SEQ ID NO 1 has previously been deposited in the Genbank under the accession number AB021878 and the corresponding protein, SEQ Id NO 2, has been deposited in the Genbank under the accession number BAA83337.

The term "essentially similar to" also includes a complement of the sequences 1 or 2; RNA, DNA, a cDNA or a genomic DNA corresponding to the sequences 1 or 2; a variant of the gene or protein due to the degeneracy of the genetic code; an allelic variant of the gene or protein; and different splice variant of the gene or protein and variants that are interrupted by one or more intervening sequences. The term "essentially similar to" also includes a family member or homologues, orthologues and paralogues of the gene or protein represented by SEQ ID NO 1 or 2. Moreover, the conservation of NHX genes among diverse prokaryotic and eukaryotic species also allows the use of non-plant NHX genes for the methods of the present invention, such as NHX genes/proteins from yeast, fungi, molds, algae, plants, insects, animals, human etc. Advantageously, nucleic acids or proteins essentially similar SEQ ID NO 1 or 2, may be used in the methods of the present invention.

According to a preferred feature of the present invention, the nucleic acid sequence capable of modulating expression of a NHX gene or modulating activity and/or levels of a NHX protein is a nucleic acid sequence homologous to SEQ ID NO 1 or encodes a protein homologous to SEQ ID NO 2. Such a homologue is preferably isolated from a plant, whether from the same plant species as SEQ ID NO 2 (*Oryza sativa*) or from a different plant species. More preferably, the homologous nucleic acid sequence is isolated from a monocotyledonous plant, further preferably from a plant belonging to the family Poaceae, most preferably from Oryzaceae. Most preferably, the nucleic acid sequence capable of modulating expression of a NHX gene or activity and/or levels of a NHX protein is a nucleic acid sequence as represented by SEQ ID NO 1 or portion of a fragment or a nucleic acid sequence encoding an amino acid sequence represented by SEQ ID NO 2 or a homologue, derivative or active fragment thereof. Preferably, the nucleic acid sequence represented by SEQ ID NO 1 is overexpressed in a plant. However, it should be clear that the applicability of the invention is not limited to use of the nucleic acid represented by SEQ ID NO 1 nor to the nucleic acid sequence encoding the amino acid sequence of SEQ ID NO 2, but that other nucleic acid sequences encoding homologues, derivatives or active fragments of SEQ ID NO 2 may be useful in the methods of the present invention.

Homologues of SEQ ID NO 1 can be found in many prokaryotic and eukaryotic organisms. The closest homologues are found in the plant kingdom. Close gene homologues are amongst others, isolated from *Hordeum vulgare* (AB089197), from *Triticum aestivum* (AY04246), *Zea Mays* (AF307944) and from *Arabidopsis* and *Atriplex* as mentioned before. Advantageously, these homologues of the rice NHX protein can be used for the methods of the present invention.

Also as part of the present invention, sequences homologous to SEQ ID NO 1 were identified in the sugarcane genome (reference codes SCRLFL4028D02.g, SEQ ID NO 3 and corresponding amino acid sequence SEQ ID NO 4 and SCRURT2005c05.g, SEQ ID NO 5 and corresponding amino acid sequence SEQ ID NO 6 and SCCCLR1066F02.g SEQ ID NO 7 and corresponding amino acid sequence SEQ ID NO 8). These sequences might be only partial and encode a partial NHX protein. Since it is in the realm of a skilled person to derive from a partial sequence a full length sequence of a gene, based on the genomic information, a further aspect of the invention are sequence comprising at least a part of the sequences as presented by any of SEQ ID NO 3 to 8, for example sequence of the full length gene and the full length protein.

As more genomes are being sequenced, it is expected that many more NHX homologues shall be identifiable.

It is clear from the high sequence identity between the sugarcanes sequences and the rice sequences (around 70-80%) that NHX is highly conserved among plant species.

Methods for the search and identification of homologues of the NHX protein would be well within the realm of a person skilled in the art. Methods for the search and identification of such homologues genes, involve screening sequence information with the sequences as provided by the present invention in SEQ ID NO 1 and 2. This sequence information is available for example in public databases, that include but are not limited to the DNA Database of Japan (DDBJ) http://www.ddbj.nig.ac.jpD; Genbank-(http://www.ncbi.nlm.nih.gov/web/Genbank/index.html); and the European Molecular Biology Laboratory Nucleic Acid Sequence Database (EMBL) (http:/w.ebi.ac.uk/ebi-docs/embl-db.html) or versions thereof or the MIPS database . A number of different search algorithms have been developed, including but not limited to the suite of programs referred to as BLAST programs. There are five implementations of BLAST, three designed for nucleotide sequence queries (BLASTN, BLASTX, and TBLASTX) and two designed for protein sequence queries (BLASTP and TBLASTN) (Coulson, Trends in Biotechnology: 76-80, 1994; Birren et al., GenomeAnalysis, 1: 543, 1997). Such methods involve alignment and comparison of sequences. The BLAST algorithm calculates percent sequence identity and performs a statistical analysis of the similarity between the two sequences. The software for performing BLAST analysis is publicly available through the National Centre for Biotechnology Information. Other such software or algorithms are GAP, BESTFIT, FASTA and TFASTA. GAP uses the algorithm of Needleman and Wunsch (J. Mol. Biol. 48: 443-453, 1970) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps.

In a particular embodiment of the present invention, the homologous genes as described above belong to the same gene family as the gene corresponding to SEQ ID NO 1. The analysis of a gene family can be carried out by sequence similarity analysis. To perform this analysis one can use standard programs for multiple alignments e.g. Clustal W. A neighbour-joining tree of the proteins homologous to NHX, gives a good overview of their structural and ancestral relationship. In the *Arabidopsis* genome a number of family members of the NHX protein were identified (NHX1, NHX2, NHX3, NHX4 and NHX5, Yokoi et al. 2002 The plant journal 30, 529-539). Therefore it is expected that also in other plant such as rice and other monocot plants, other family members of the NHX protein will be identified. Advantageously also these family members are useful in the methods of the present invention. In particular and according to a preferred embodiment of the present invention, the method for modifying plant growth characteristics involves the modulation of expression of a gene encoding a NHX1 gene

and/or the modulation of activity and/or the level of a NHX1 protein. Herein, a NHX1 protein has more sequence homology with the OsNHX protein as presented in SEQ ID NO 2, than with another rice (NHX) protein. Since no other rice NHX proteins are identified in the rice genome today, an NHX1 protein as used herein refers for the moment also to a protein with more sequence identity with the NHX1 protein from *Arabidopsis* than with any of the others NHX2, NHX3, NHX4 or NHX5 from *Arabidopsis*. Sequence identity can be calculated with an alignment program as described above, for example with the program align X as a module of the VNTi suite 5,5 software package, using the standard parameters.

These above-mentioned analyses for sequence homology can be done on the full-length sequence or based on a comparison of certain regions such as conserved domains. The identification of such domains, would also be well within the realm of the person skilled in the art and involve for example, a computer readable format of the nucleic acids of the present invention, the use of alignment software programs and the use of publicly available information on protein domains, conserved motifs and boxes. This information is available in the PRODOM (http://www.biochem.ucl.ac.uk/bsm/dbbrowser/jj/prodomsrchjj.html), PIR (http://pir.georgetown.edu/) or pFAM (http://pfam.wustl.edu/) database. Sequence analysis programs designed for motif searching can be used for identification of fragments, regions and conserved domains as mentioned above. Preferred computer programs would include but are not limited to MEME, SIGNALSCAN, and GENESCAN. A MEME algorithm (Version 2.2) can be found in version 10.0 of the GCG package; or on the Internet site http://www.sdsc.edu/MEME/meme. SIGNALSCAN version 4.0 information is available on the Internet site http://biosci.cbs.umn.edu/software/sigscan.html. GENESCAN can be found on the Internet site http://gnomic.stanford.edu/GENESCANW.html.

It is obvious for the person skilled in the art that the sequences as now provided by the present invention, i.e. sequences essentially similar to any of SEQ ID NO 3 to 8 can easily be used to find similar sequence in other species and other organisms. Also a sequence as provided in the present invention, for example the promoter gene combination, for example the sequence as presented in SEQ ID NO11, can be used to design and mimic experiments to alter growth characteristics of a plant. Therefore, in one application of the invention, a nucleic acid or protein of the invention, preferably a plant gene or protein essentially similar to any of SEQ ID NO 3 to 8 or essentially similar to SEQ ID NO 11 can be recorded on a computer readable media. As used herein "computer readable media" refers to any medium that can be read and accessed directly by a computer. Such media include, but are not limited to magnetic storage media, such as floppy disc, hard disc, storage medium and magnetic tape, optical storage media such as CD-ROM, electrical storage media such as RAM or ROM. These readable

formats will allow a skilled person to find homologues of the sequences SEQ ID NO 1 to 8, 11 in other plant species or other organisms.

The term NHX protein includes proteins homologous to SEQ ID NO 2. "Homologues" of a protein according to SEQ ID NO 2 encompass, peptides, oligopeptides, polypeptides, proteins and enzymes having amino acid substitutions, deletions and/or insertions relative to the unmodified protein in question and having similar biological and functional activity as the unmodified protein according to SEQ ID NO 2 from which they are derived. To produce such homologues, amino acids of the protein may be replaced by other amino acids having similar properties (such as similar hydrophobicity, hydrophilicity, antigenicity, propensity to form or break α-helical structures or β-sheet structures). Conservative substitution tables are well known in the art (see for example Creighton (1984) Proteins. W.H. Freeman and Company). "Homologues" of a nucleic acid or according to SEQ ID NO 1 encompass nucleic acids having nucleotide substitutions, deletions and/or insertions relative to the unmodified nucleic acid in question and having similar biological and functional activity as the unmodified nucleic acid according to SEQ ID NO 1 from which they are derived. The homologues useful in the methods according to the invention have a percentage of identity with a value that is equal to any value lying between 60% and 99.99%. For example, they have at least 50% sequence identity or similarity (functional identity) to the unmodified nucleic acid/protein, alternatively at least 60% sequence identity or similarity to an unmodified nucleic acid/protein. Alternatively there is at least 70% sequence identity or similarity to an unmodified nucleic acid/protein. Typically, the homologues have at least 80% sequence identity or similarity to an unmodified nucleic acid/protein, preferably at least 85% sequence identity or similarity, further preferably at least 90% sequence identity or similarity to an unmodified protein, most preferably at least 95%, 96%, 97%, 98% or 99% sequence identity or similarity to an unmodified nucleic acid/protein. Therefore the expression "essentially similar to" as used herein also means having a percentage of sequence identity or sequence similarity as mentioned above. The percentage of identity can be calculated by using an alignment program as mentioned above. For example, the percentage of identity can be calculated using the program GAP or the program align X, as a module of the vector NTI suite 5.5 software package, using the standard parameters (for example GAP penalty 5, GAP opening penalty 15, GAP extension penalty 6.6). There is 71% sequence identity between the AtNHX1 protein and the OsNHX1 protein. Therefore, suitable for use in the methods of the present invention are proteins that are at least 71% homologous to the protein as presented in SEQ ID NO 2.

Two special forms of homology, orthologous and paralogous, are evolutionary concepts used to describe ancestral relationships of genes/proteins. The term "paralogous" relates to gene-

duplications within the genome of a species leading to paralogous genes. The term "orthologous" relates to homologous genes in different organisms due to ancestral relationship. The term "homologues" as used herein also encompasses paralogues and orthologues of the proteins useful in the methods according to the invention.

"Substitutional variants" of a NHX protein are those in which at least one residue in an amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues, and deletions will range from about 1-20 residues. Preferably, amino acid substitutions comprise conservative amino acid substitutions.

"Insertional variants" of a NHX protein are those in which one or more amino acid residues are introduced into a predetermined site in a protein. Insertions can comprise amino-terminal and/or carboxy-terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid sequence will be smaller than amino- or carboxy-terminal fusions, of the order of about 1 to 10 residues. Examples of amino- or carboxy-terminal fusion proteins or peptides include the binding domain or activation domain of a transcriptional activator as used in the yeast two-hybrid system, phage coat proteins, (histidine)$_6$-tag, glutathione S-transferase-tag, protein A, maltose-binding protein, dihydrofolate reductase, Tag•100 epitope, c-myc epitope, FLAG®-epitope, lacZ, CMP (calmodulin-binding peptide), HA epitope, protein C epitope and VSV epitope.

"Deletion variants" of a NHX protein are characterised by the removal of one or more amino acids from the protein. Amino acid variants of a protein may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce substitution, insertion or deletion variants of a protein are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA are well known to those skilled in the art and include M13 mutagenesis, T7-Gen *in vitro* mutagenesis (USB, Cleveland, OH), QuickChange Site Directed mutagenesis (Stratagene, San Diego, CA), PCR-mediated site-directed mutagenesis or other site-directed mutagenesis protocols.

The term "derivatives" of a NHX protein refers to peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise substitutions, or deletions or additions of naturally and non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of the protein as presented in SEQ ID NO 2. "Derivatives" of a protein

as presented in SEQ ID NO 2 encompass peptides, oligopeptides, polypeptides, proteins and enzymes which may comprise naturally occurring altered, glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring form of the polypeptide. A derivative may also comprise one or more non-amino acid substituents compared to the amino acid sequence from which it is derived, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which is bound to facilitate its detection, and non-naturally occurring amino acid residues relative to the amino acid sequence of a naturally-occurring protein.

"Active fragments" of a NHX protein encompasses at least five contiguous amino acid residues of a protein, which residues retain similar biological and/or functional activity to the naturally occurring protein. A preferred fragment of a NHX protein is a C-terminal truncated version of the NHX protein, lacking one or more or all of the 100 last amino acids. Other preferred fragments are fragments of the NHX protein starting at the second or third or further internal methionin residues.

Advantageously, the method according to the present invention may also be practised using fragments of DNA or of a nucleic acid sequence. The term "DNA fragment or DNA segment" refers to a piece of DNA derived or prepared from an original (larger) DNA molecule, which DNA fragment or segment, when expressed in a plant, gives rise to plants having modified growth characteristics. The DNA fragment or segment may comprise many genes, with or without additional control elements, or may contain just spacer sequences etc.

One particular fragment of DNA that comprises a NHX encoding gene is the nucleic acid as presented in SEQ ID NO 3, 5 or 7. In a preferred embodiment of the invention, these nucleic acids are introduced into a plant to alter growth characteristics.

A NHX gene suitable for the methods of the present invention also encompasses a nucleic acid capable of hybridising with SEQ ID NO 1. The present invention thus also encompasses nucleic acid sequences capable of hybridising with a nucleic acid sequence encoding a NHX protein, which nucleic acid sequences may also be useful in practising the methods according to the invention. The term "hybridisation" as defined herein is the process wherein substantially homologous complementary nucleotide sequences anneal to each other. The hybridisation process can occur entirely in solution, i.e. both complementary nucleic acids are in solution. Tools in molecular biology relying on such a process include the polymerase chain reaction (PCR; and all methods based thereon), subtractive hybridisation, random primer extension,

nuclease S1 mapping, primer extension, reverse transcription, cDNA synthesis, differential display of RNAs, and DNA sequence determination. The hybridisation process can also occur with one of the complementary nucleic acids immobilised to a matrix such as magnetic beads, Sepharose beads or any other resin. Tools in molecular biology relying on such a process include the isolation of poly (A+) mRNA. The hybridisation process can furthermore occur with one of the complementary nucleic acids immobilised to a solid support such as a nitro-cellulose or nylon membrane or immobilised by e.g. photolithography to e.g. a siliceous glass support (the latter known as nucleic acid arrays or microarrays or as nucleic acid chips). Tools in molecular biology relying on such a process include RNA and DNA gel blot analysis, colony hybridisation, plaque hybridisation, *in situ* hybridisation and microarray hybridisation. In order to allow hybridisation to occur, the nucleic acid molecules are generally thermally or chemically denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridisation is influenced by conditions such as temperature, salt concentration and hybridisation buffer composition. High stringency conditions for hybridisation include high temperature and/or low salt concentration (salts include NaCl and Na_3-citrate) and/or the inclusion of formamide in the hybridisation buffer and/or lowering the concentration of compounds such as SDS (detergent) in the hybridisation buffer and/or exclusion of compounds such as dextran sulphate or polyethylene glycol (promoting molecular crowding) from the hybridisation buffer. Conventional hybridisation conditions are described in, for example, Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York, but the skilled craftsman will appreciate that numerous different hybridisation conditions can be designed in function of the known or the expected homology and/or length of the nucleic acid sequence. With specifically hybridising is meant hybridising under stringent conditions. Specific conditions for "specifically hybridising" are for example: hybridising under stringent conditions such as a temperature of 60°C followed by washes in 2XSSC, 0.1XSDS, and 1X SSC, 0.1X SDS. Sufficiently low stringency hybridisation conditions are particularly preferred to isolate nucleic acids heterologous to the DNA sequences of the invention defined supra. Elements contributing to heterology include allelism, degeneration of the genetic code and differences in preferred codon usage. Therefore the expression "essentially similar to" as used herein also encompasses "hybridising with" or "specifically hybridising with", as illustrated above.

The methods according to the present invention may also be practiced using an alternative splice variant of a nucleic acid sequence encoding a NHX protein, for example a splice variant of SEQ ID NO 1. The term "alternative splice variant" as used herein encompasses variants of a nucleic acid sequence in which introns and/or exons have been excised and/or replaced and/or added. Such variants will be ones in which the biological activity of the protein remains

unaffected, which can be achieved by selectively retaining functional segments of the protein. Such splice variants may be found in nature or may be manmade. Methods for making such splice variants are well known in the art. Therefore according to another aspect of the present invention, there is provided, a method for modifying the growth characteristics, comprising modulating expression in a plant of a nucleic acid sequence encoding an alternative splice variant of a NHX protein and/or by modulating activity of a protein encoded by the splice variant of a NHX protein.

Another method for modifying plant growth characteristics resides in the use of allelic variants of a gene essentially similar to SEQ ID NO 1. Allelic variants exist in nature and encompassed within the methods of the present invention is the use of these natural alleles. "Allelic variants" are defined as to comprise single nucleotide polymorphisms (SNPs) as well as small insertion/deletion polymorphisms (INDELs; the size of INDELs is usually less than 100 bp). SNPs and INDELs form the largest set of sequence variants in naturally occurring polymorphic strains of most organisms. Differences between alleles are naturally occurring differences between the genes of different plants of the same species. These differences can be substitution and/or addition and/or deletion of for example 1, 2, 3 or more base pairs. Additionally or alternatively, in particular conventional breeding programs, such as for example marker assisted breeding, it is sometimes practical to introduce allelic variation in the plants by mutagenic treatment of a plant. One suitable mutagenic method is EMS mutagenesis. Identification of allelic variants then takes place by, for example, PCR. This is followed by a selection step for selection of superior allelic variants of the sequence in question and which give rise to altered growth characteristics. Selection is typically carried out by monitoring growth performance of plants containing different allelic variants of the sequence in question (for example SEQ ID NO 1). Monitoring growth performance can be done in a greenhouse or in the field. Further optional steps include crossing plants in which the superior allelic variant was identified with another plant. This could be used, for example, to make a combination of interesting phenotypic features. According to another aspect of the present invention, advantage may be taken of the nucleotide sequence capable of modulating expression of a nucleic acid encoding a NHX protein or capable of modulating the activity of a protein essentially similar as SEQ ID NO 2 in breeding programs. The nucleic acid sequence may be on a chromosome, or a part thereof, chromosomes, comprising at least the nucleic acid sequence encoding the NHX protein and preferably also one or more related family members. These breeding programs can be conventional breeding programs or marker assisted breeding programs. For example, in such a program, a DNA marker is identified which may be genetically linked to the gene capable of modulating the activity NHX protein in a plant (which gene can be the gene encoding a NHX protein or another gene capable of influencing the

activity and/or levels of a NHX protein). This DNA marker is then used in breeding programs to select plants having altered growth characteristics.

The methods according to the present invention may also be practised by introducing into a plant at least a part of a (natural or artificial) chromosome (such as a Bacterial Artificial Chromosome (BAC)), which chromosome contains at least a gene/nucleic acid sequence encoding a NHX protein (such as for example a protein represented by SEQ ID NO 2), preferably together with one or more related gene family members. Therefore, according to a further aspect of the present invention, there is provided a method for modifying the growth characteristics of plants by introducing into a plant at least a part of a chromosome comprising at least a gene/nucleic acid encoding a NHX protein.

The sequences presented by SEQ ID NO 3 or 5 or 75 were hitherto unknown as a NHX encoding genes. Therefore, there is provided as a further aspect of the present invention, a nucleic acid encoding a NHX protein. These genes were isolated from sugarcane and can be used for the methods of the present invention. Accordingly, the present invention encompasses an isolated nucleic acid, encoding NHX like protein or a fragment thereof, selected from the group consisting of:

a. a nucleic acid sequence encoding an amino acid sequence as presented in SEQ ID NO 4 or 6 or 8;
b. a nucleic acid sequence depicted in SEQ ID NO 3 or 5 or 7, or the complementary strand thereof;
c. nucleic acid sequences which are degenerated as a result of the genetic code to the nucleic acid sequences defined in (a) or (b);
d. nucleic acid sequences which are allelic variants to the nucleic acid sequences defined in (a) or (b);
e. nucleic acid sequences which hybridise, preferably under stringent conditions, to sequences defined in (a), (b), (c) or (d);
f. nucleic acid sequences which have 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to any one or more of the sequence defined in (a), (b), (c) or (d)
g. nucleic acid sequences encoding a protein which has 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to any one or more from the sequences defined in SEQ ID NO 4, 6 or 8;
h. nucleic acid sequences encoding a fragment of a protein encoded by a nucleic acid of any one of (a) to (g).

In another embodiment, the present invention provides an isolated NHX protein comprising at least part of one of the polypeptides selected from the group consisting of:

a) a polypeptide as given in SEQ ID NO 4, 6 or 8,
b) a polypeptide with an amino acid sequence which has at least 50% sequence identity, preferably 60%, 70% sequence identity; more preferable 80% or 90% sequence identity, most preferable 95%, 96%, 97%, 98% or 99% sequence identity to any one or more of the amino acid sequence as given in SEQ ID NO 4, 6 or 8,
c) a polypeptide encoded by a nucleic acid as mentioned above or a homologue, a derivative, an immunologically active and/or functional fragment of a protein as defined in any of (a) to (d).

According to a more preferred embodiment of the present invention there is provided a plant nucleic acid as described above or a plant protein as described above, wherein said nucleic acid is not encoding and/or wherein said protein is not the *Arabidopsis* NHX for example protein NHX1 to NHX5, or not the rice NHX1 protein BAA83337 or AP004274, or not a NHX from *Atriplex* as mentioned before, or not NHX from *Hordeum vulgare* (for example AB089197), not from *Triticum aestivum* (for example AY04246), not from *Zea Mays* (for example AF307944 or AY110110), or not from *Gossypium hirsutum*.

According to a further aspect of the present invention, genetic constructs and vectors to facilitate the introduction and/or expression and/or maintenance of the nucleotide sequence capable of modulating expression of a nucleic acid encoding a NHX protein and promoter into a plant cell, tissue or organ are provided. Therefore, according to a further aspect of the present invention, there is provided a genetic construct comprising:

(i) a nucleic acid sequence capable of modulating expression of a nucleic acid encoding a NHX protein and/or capable of modulating the activity of NHX protein;
(ii) a control sequence capable of regulating expression of the nucleic acid sequence of (i); and optionally
(iii) a transcription termination sequence.

The genetic construct can be an expression vector wherein said nucleic acid sequence is operably linked to one or more control sequences allowing expression in prokaryotic and/or eukaryotic host cells.

According to a preferred embodiment of the invention, the genetic construct is an expression vector designed to overexpress the nucleic acid sequence according to a). In this construct a gene essentially similar to SEQ ID NO 1, for example the sequence as represented by SEQ ID

NO 1 (or one or more parts thereof) is cloned in the sense orientation relative to the promoter sequence. According to a further preferred embodiment this promoter is a tissue-specific promoter, further preferably a seed-specific promoter, more preferably an endosperm-specific promoter, most preferably a prolamin promoter, such as for example a rice prolamin promoter. Therefore according to a particular embodiment of the invention there is provided a genetic construct comprising a tissue-specific promoter, preferably as a seed-specific promoter, most preferably an endosperm-specific promoter, and comprising an NHX encoding gene. Accordingly, in one particular embodiment of the invention, the vector construct carries an expression cassette essentially similar to an expression cassette as presented in SEQ ID NO 11, comprising the rice prolamin promoter, the rice NHX1 gene and the T-zein + T-rubisco transcription terminator sequence. "Sequences essentially similar to SEQ ID NO 11" means sequences that have another rice prolamin promoter or a prolamin promoter from another species or another endosperm-specific promoter or another promoter with similar expression patterns as the rice prolamin promoter. "Sequences essentially similar to SEQ ID NO 11" also means sequences comprising a NHX sequence hybridising with SEQ ID NO NO 1 or encoding a protein homologous to SEQ ID NO 2. "Essentially similar to SEQ ID NO 11" also means comprising another transcription terminator sequence.

Therefore according to another aspect of the invention, there is provided an isolated nucleic acid, encoding NHX like protein or a fragment thereof, selected from the group consisting of:

(a) a nucleic acid sequence depicted in SEQ ID NO 11, or the complementary strand thereof;
(b) nucleic acid sequences which are degenerated as a result of the genetic code to the nucleic acid sequences defined in (a);
(c) nucleic acid sequences which are allelic variants to the nucleic acid sequences defined in (a);
(d) nucleic acid sequences which hybridise, preferably under stringent conditions, to sequences defined in (a)
(e) nucleic acids which have at least part of an prolamin promoter operably coupled to at least part of an NHX gene.

Alternatively, the genetic constructs can be aimed at silencing the gene expression of the nucleic acid of a) and may comprise the nucleotide sequence essentially similar to SEQ ID NO 1, a sense and/or antisense orientation relative to the promoter sequence. The sense or antisense copies of at least part of the endogenous gene in the form of direct or inverted repeats may be utilised in the methods according to the invention. The growth of plants may also be modified by introducing into a plant at least part of an antisense version of the

nucleotide sequence represented by SEQ ID NO 1. It should be clear that part of the nucleic acid could achieve the desired result. Homologous anti-sense genes are preferred to heterologous anti-sense genes, homologous genes being plant genes, preferably plant genes from the same plant species, and heterologous genes being genes from non-plant species.

In the present invention, plants are transformed with a genetic construct, such as an expression vector, comprising the sequence of interest (i.e. the nucleic acid sequence capable of modulating expression of nucleic acid encoding a NHX protein and/or capable of modulating the activity and/or levels of a NHX protein), which is operably linked to a promoter. Advantageously, any type of promoter may be used to drive expression of the nucleic acid sequence depending on the desired outcome. For example, a meristem-specific promoter, such as the RNR (ribonucleotide reductase), cdc2a promoter and the cyc07 promoter, could be used to effect expression in all growing parts of the plant, thereby increasing cell proliferation, which in turn increases yield, harvest index or biomass. If the desired outcome would be to (further) influence seed characteristics, such as the storage capacity, seed size, seed number, biomass etc., then a seed-specific promoter, such as p2S2, pPROLAMIN, pOLEOSIN could be selected. An aleurone-specific promoter may be selected in order to increase growth at the moment of germination, thereby increasing the transport of sugars to the embryo. An inflorescence-specific promoter, such as pLEAFY, may be utilised if the desired outcome would be to modify the number of flower organs. To produce male-sterile plants one would need an anther specific promoter. To impact on flower architecture for example petal size, one could choose a petal-specific promoter. If the desired outcome would be to modify growth and/or developmental characteristics in particular organs, then the choice of the promoter would depend on the organ to be modified. For example, use of a root-specific promoter would lead to increased growth and/or increased biomass or yield of the root and/or phenotypic alteration of the root. This would be particularly important where it is the root itself that is the desired end product, such crops including sugar beet, turnip, carrot, and potato. A fruit-specific promoter may be used to modify, for example, the strength of the outer skin of the fruit or to increase the size of the fruit. A green tissue-specific promoter may be used to increase leaf size. A cell wall-specific promoter may be used to increase the rigidity of the cell wall, thereby increasing resistance towards pathogens. An anther-specific promoter may be used to produce male-sterile plants. A vascular-specific promoter may be used to increase transport from leaves to seeds. A nodule-specific promoter may be used to increase the nitrogen fixing capabilities of a plant, thereby increasing the nutrient levels in a plant.
Also constitutive promoters, such as the GOS2 promoter or promoters with a similar strong and ubiquitous expression pattern, are particularly useful in the methods of the present invention.

Most preferably, in the construct of the present invention and in the methods of the present invention, the nucleic acid sequence capable of modulating expression of a gene encoding a NHX protein is operably linked to a tissue-specific promoter, more preferably to a seed-specific promoter, most preferably to an endosperm-specific promoter, such as for example the prolamin promoter. The term "tissue-specific" promoter as defined herein refers to a promoter that is expressed predominantly in at least one tissue or organ. Preferably the tissue-specific promoter is a prolamin promoter, with the predominant expression level in the endosperm, or a promoter of similar strength and/or a similar expression pattern. Similar strength and/or similar expression pattern can be analysed for example by coupling the promoters to a reporter gene and check the function of the reporter gene in tissues of the plant. One suitable reporter gene is beta-glucuronidase and the colorimertic GUS staining to visualize the reporter gene activity in a plant tissue is well known to a person skilled in the art.

Examples of other seed-specific promoters are presented in table 1, and these promoters or derivatives thereof are useful for the methods of the present invention.

Table 1

Exemplary constitutive promoters for use in the performance of the present invention

Gene source	Expression pattern	Reference
seed-specific genes	seed	Simon, *et al., Plant Mol. Biol. 5:* 191, 1985; Scofield, *et al., J. Biol. Chem. 262:* 12202, 1987.; Baszczynski, *et al., Plant Mol. Biol. 14:* 633, 1990.
Brazil Nut albumin	seed	Pearson, et al., Plant Mol. Biol. 18: 235-245, 1992.
legumin	seed	Ellis, et al., Plant Mol. Biol. 10: 203-214, 1988.
glutelin (rice)	seed	Takaiwa, et al., Mol. Gen. Genet. 208: 15-22, 1986; Takaiwa, et al., FEBS Letts. 221: 43-47, 1987.
zein	seed	Matzke et al Plant Mol Biol. 14(3):323-32 1990
napA	seed	Stalberg, *et al, Planta 199:* 515-519, 1996.
wheat LMW and HMW glutenin-1	endosperm	Mol Gen Genet 216:81-90, 1989; NAR 17:461-2, 1989
wheat SPA	seed	Albani *et al,* Plant Cell, 9: 171-184, 1997
wheat α, β, γ-gliadins	endosperm	EMBO 3:1409-15, 1984
barley *ltr1* promoter	endosperm	
barley B1, C, D, hordein	endosperm	Theor Appl Gen 98:1253-62, 1999; Plant J 4:343-55, 1993; Mol Gen Genet 250:750-60, 1996
barley DOF	endosperm	Mena *et al,* The Plant Journal, 116(1): 53-62, 1998
blz2	endosperm	EP99106056.7
synthetic promoter	endosperm	Vicente-Carbajosa *et al., Plant J. 13:* 629-640, 1998.

rice prolamin NRP33	endosperm	Wu *et al*, Plant Cell Physiology 39(8) 885-889, 1998
rice □-globulin Glb-1	endosperm	Wu *et al*, Plant Cell Physiology 39(8) 885-889, 1998
rice OSH1	embryo	Sato *et al*, Proc. Natl. Acad. Sci. USA, 93: 8117-8122, 1996
rice □-globulin REB/OHP-1	endosperm	Nakase *et al*. Plant Mol. Biol. 33: 513-522, 1997
rice ADP-glucose PP	endosperm	Trans Res 6:157-68, 1997
maize ESR gene family	endosperm	Plant J 12:235-46, 1997
sorgum □-kafirin	endosperm	PMB 32:1029-35, 1996
KNOX	embryo	Postma-Haarsma *et al*, Plant Mol. Biol. 39:257-71, 1999
rice oleosin	embryo and aleuron	Wu *et al*, J. Biochem., 123:386, 1998
sunflower oleosin	seed (embryo and dry seed)	Cummins, *et al*., Plant Mol. Biol. 19: 873-876, 1992
PRO0117, putative rice 40S ribosomal protein	weak in endosperm	
PRO0135, rice alpha-globulin	strong in endosperm	
PRO0136, alanine aminotransferase	weak in endosperm	
PRO0147, trypsin inhibitor ITR1 (barley)	weak in endosperm	
PRO0151, rice WSI18	embryo + stress	
PRO0175, rice RAB21	embryo + stress	
PRO0218, rice oleosin 18kd	aleurone + embryo	

The terms "regulatory element", "control sequence", "promoter" are all used herein interchangeably and taken in a broad context refer to regulatory nucleic acid sequences capable of effecting expression of the sequences to which they are ligated. Encompassed by the aforementioned terms are transcriptional regulatory sequences derived from a classical eukaryotic genomic gene (including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence) and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. Also included within the term is a transcriptional regulatory sequence of a classical prokaryotic gene, in which case it may include a −35 box sequence and/or −10 box transcriptional regulatory sequences. The term "regulatory element" also encompasses a synthetic fusion molecule or derivative, which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ. The terms "control sequence", "regulatory sequence", "regulatory element" and "promoter" are used interchangeably herein. The term "operably linked" as used herein refers to a functional linkage between the promoter sequence and the gene of interest, such that the promoter sequence is able to initiate transcription of the gene of interest.

Optionally, one or more terminator sequences may also be used in the construct introduced into a plant. The term "terminator" encompasses a control sequence which is a DNA sequence

at the end of a transcriptional unit which signals 3' processing and polyadenylation of a primary transcript and termination of transcription. Additional regulatory elements may include transcriptional as well as translational enhancers. Those skilled in the art will be aware of terminator and enhancer sequences, which may be suitable for use in performing the invention. Such sequences would be known or may readily be obtained by a person skilled in the art.

The genetic constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type, for example a bacterial cell, when said genetic construct is required to be maintained as an episomal genetic element (e.g. plasmid or cosmid molecule) in a cell. Preferred origins of replication include, but are not limited to, the f1-ori and colE1 origins of replication.

The genetic construct may optionally comprise a selectable marker gene. As used herein, the term "selectable marker gene" includes any gene which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof. Suitable markers may be selected from markers that confer antibiotic or herbicide resistance. Cells containing the recombinant DNA will thus be able to survive in the presence of antibiotic or herbicide concentrations that kill untransformed cells. Examples of selectable marker genes include the *bar* gene which provides resistance to the herbicide Basta; the neomycin phosphotransgferase (nptII) gene which confers resistance to the antibiotic kanamycin; the hygromycin phosphotransferase (hptII) gene which confers hygromycin resistance. Visual markers, such as the Green Fluorescent Protein (GFP) (Haseloff *et al.*, Proc. Natl. Acad. Sci. U.S.A, 94 (6), 2122-2127, 1997) and luciferase may also be used as selectable markers. An entire plant may be generated from a single transformed plant cell through cell culturing techniques known to those skilled in the art. Other suitable selectable marker genes include the ampicillin resistance (Amp^r), tetracycline resistance gene (Tc^r), bacterial kanamycin resistance gene (Kan^r), phosphinothricin resistance gene, β-glucuronidase (GUS) gene, and chloramphenicol acetyltransferase (CAT) gene amongst others.

Recombinant DNA constructs for use in the methods according to the present invention may be constructed using recombinant DNA technology well known to persons skilled in the art. The gene constructs may be inserted into vectors, which may be commercially available, suitable for transforming into plants and suitable for expression of the gene of interest in the transformed cells. The present invention also relates to a method for the production of transgenic plants, plant cells or plant tissues, comprising introduction of a nucleic acid

molecule of the invention in an expressible format or a vector as defined above into a plant, plant cell or plant tissue. Methods for effecting the activity and/or levels of a NHX protein or a homologue or derivative thereof as defined in the current invention in a plant cell, tissue or organ, include either the introduction of the protein directly into said cell, tissue or organ such as by microinjection of ballistic means or, alternatively, (stable or transient) introduction of an isolated nucleic acid molecule encoding said protein in an expressible format into the genome of a plant cell. The nucleic acid can be operably linked to one or more control sequences or can be integrated in a vector according to the invention and/or can be stably integrated into the genome of a plant cell.

The nucleic acid molecule or a genetic construct according to the invention, for example comprising the NHX encoding gene, may be introduced into a cell using any known method for the transfection or transformation of a cell. A whole organism may be regenerated from a single transformed or transfected cell, using methods known in the art. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a genetic construct of the present invention and a whole plant regenerated there from. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

The gene of interest is preferably introduced into a plant by transformation. The term "transformation" as referred to herein encompasses the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for transfer. The polynucleotide may be transiently or stably introduced into a host cell and may be maintained non-integrated, for example, as a plasmid, or alternatively, may be integrated into the host genome. The resulting transformed plant cell can then be used to regenerate a transformed plant in a manner known to persons skilled in the art. Transformation of a plant species is now a fairly routine technique. Advantageously, any of several transformation methods may be used to introduce the gene of interest into a suitable ancestor cell. Transformation methods include the use of liposomes, electroporation, chemicals that increase free DNA uptake, injection of the DNA directly into the plant, particle gun bombardment, transformation using viruses or pollen and microprojection. Methods may be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. *et al.*, Nature 296, 72-74, 1982; Negrutiu I. *et al.*, Plant Mol. Biol. 8, 363-373, 1987); electroporation of protoplasts (Shillito R.D. *et al.* Bio/Technol 3, 1099-1102, 1985); microinjection into plant material (Crossway A. *et al.*, Mol. Gen Genet 202, 179-185,

1986); DNA or RNA-coated particle bombardment (Klein T.M. *et al.*, Nature 327, 70, 1987) infection with (non-integrative) viruses and the like. A preferred method according to the present invention comprises the protocol according to Hiei *et al.*, Plant J., 6 (2), 271-282, 1994 in the case of rice transformation. For corn transformation, a preferred method according to the present invention, comprises the agrobacterium-based transformation of an immature embryo as described in EP0604662, EP0672752, EP0971578, EP0955371 or EP0558676 or following the protocol of Frame et al. (*Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system.* Plant Physiol. 2002 May;129(1):13-22.

Generally after transformation, plant cells or cell groupings are selected for the presence of one or more markers which are encoded by plant-expressible genes co-transferred with the gene of interest, following which the transformed material is regenerated into a whole plant. Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the gene of interest, copy number and/or genomic organisation. Alternatively or additionally, expression levels of the newly introduced DNA may be undertaken using Northern and/or Western analysis, both techniques being well known to persons having ordinary skill in the art.

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformants, and the T2 plants further propagated through classical breeding techniques.

The generated transformed organisms may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed rootstock grafted to an untransformed scion).

The present invention also provides plants with improved growth characteristics compared to the wild-type plant when grown in normal growth conditions, obtainable by the method according to the present invention, which plants have improved growth characteristics when compared to the wild-type plant grown under normal growth conditions and which plants have altered expression levels of a nucleic acid encoding a NHX protein. Preferably said plants are transformed with a NHX encoding gene under the control of a seed-specific promoter.

By the present invention, there is also provided a method for the production of a transgenic plant and for modifying the growth characteristics of a plant grown under non)stress groth conditions, which method comprises:

(i) introducing into a plant cell a nucleic acid sequence capable of modulating expression of a gene encoding a NHX protein and/or capable of modulating the activity and/or levels of a NHX protein;

(ii) cultivating said plant cell under conditions promoting regeneration and mature plant growth.

Preferably is this method the genes is under the control of a tissue-specific promoter, preferable and endosperm-specific promoter.

The invention also includes host cells containing an isolated nucleic acid molecule encoding a NHX protein, preferably wherein the protein is a plant NHX protein, such as for example the protein as presented in SEQ ID NO 2, 4, 6 or 8. Preferably, in these host cells, the NHX encoding gene is in the sense orientation and under the control of a tissue specific promoter, preferably an endosperm-specific promoter. The present invention extends to a transgenic plant with altered growth characteristics, containing an isolated nucleic acid molecule encoding a NHX protein, preferably wherein the protein is as presented in SEQ ID NO 2, 4, 6 or 8. Preferred host cells according to the invention are plant cells. The present invention clearly extends to any plant or plant cell produced by any of the methods described herein, and to all plant parts and propagules thereof. The present invention extends further to encompass the progeny of a primary transformed or transfected cell, tissue, organ or whole plant that has been produced by any of the aforementioned methods, the only requirement being that progeny exhibit the same genotypic and/or phenotypic characteristic(s) as those produced in the parent by the methods according to the invention. The invention also extends to harvestable parts of a plant such as but not limited to seeds, leaves, flowers, fruits, stem cultures, stem, rhizomes, roots, tubers and bulbs.

The term "plant" as used is any of the above mentioned paragraphs describing the methods, nucleic acids, proteins or transformed plants of the present invention, herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers), and plant cells, tissues and organs. The term "plant" also therefore encompasses suspension cultures, embryos, meristematic regions, callus tissue, leaves, seeds, roots, shoots, gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily *Viridiplantae*, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list

comprising *Acacia spp., Acer spp., Actinidia spp.,Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chaenomeles spp.,Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Diheteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehrartia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Fagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii, Ginkgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incarnata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp.Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea Mays*, amaranth, artichoke, asparagus, broccoli, Brussels sprout, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugar beet, sugar cane, sunflower, tomato, squash, and tea, trees and algae amongst others. According to a preferred feature of the present invention, the plant is a crop plant such as rice, maize, wheat, barley, soybean, sunflower, canola, sugarcane, alfalfa, millet, barley, rapeseed and cotton. Further preferably, the plant according to the present invention is a monocotyledonous plant, most preferably a cereal.

Advantageously, performance of the methods according to the present invention leads to plants having modified plant growth and/or having various modified growth characteristics, such as modified growth, modified yield/biomass and modified architecture each relative to the corresponding wild-type plants and when grown under non-saltstress conditions.

The term "modified plant growth" as used herein encompasses, but is not limited to, a faster rate of growth in one or more parts of a plant (including green biomass and including seeds), at one or more stages in the life cycle of a plant, and/or enhanced vigour, each relative to corresponding wild-type plants and grown under non-saltstress growing conditions.

The term "modified yield", preferably "increased yield" encompasses an increase in biomass in one or more parts of a plant relative to the biomass of corresponding wild-type plants and when grown under non-saltstress growing conditions. The term also encompasses an increase in seed yield, which includes an increase in the biomass of the seed and/or an increase in the number of (filled) seeds and/or in the size of the seeds and/or an increase in seed volume, each relative to corresponding wild-type plants and when grown under non-saltstress growing conditions. An increase in seed size and/or volume may also influence the composition of the seed, for example, they may contain more starch or more oil and grown and produced under non-saltstress growing conditions. An increase in seed yield could be due to an increase in the number and/or size of flowers. An increase in seed yield might also increase the harvest index, which is expressed as the ration of the total biomass over the yield of harvestable parts, such as the seeds of a cereal.

Increased yield/biomass refers to a better performance of a plant under non-saltstress conditions compared to the performance of a wild-type plant.

According to a preferred feature of the present invention, performance of the methods according to the present invention result in plants having modified yield. Preferably the modified yield includes, an increase in number of filled seeds, an increase in total number of seeds, an increase in the total seed weight, an increase in thousand kernel weight, and increase in harvest index and an increase in the number of panicles each relative to the corresponding wild-type plants. Therefore, according to the invention, there is provided a method for increasing yield of plants, which method comprises modulating expression of a nucleic acid sequence encoding a NHX protein and/or modulating the activity and/or levels of a NHX protein in a plant. Preferably in this method the NHX protein is encoded by a nucleic acid as represented by SEQ ID NO 1, 3, 5 or 7 or a portion or fragment thereof, or a sequence capable of hybridizing therewith or wherein the NHX protein is as represented by SEQ ID NO 2, 4, 6 or 8 or a homologue, derivative or active fragment thereof. Alternatively, the NHX may

be encoded by an expression cassette comprising a seed-specific promoter, for example an expression cassette as presented in SEQ ID NO 11.

The methods of the present invention are particularly favourable to be applied to crop plants, preferably cereals, because the methods of the present invention are used to increase the seed yield, thousand kernel weight and harvest index of the plant. Accordingly, a particular embodiment of the present invention relates to a method to increase seed yield and/or to increase harvest index of a cereal, comprising modifying expression of a nucleic acid essential similar to SEQ ID NO 1, 3, 5 or 7 or by introducing into a plant an NHX gene operably linked to a promoter, for example a seed-specific promoter, for example an NH gene in an expression cassette as presented in SEQ ID NO 11 and by growing the transformed plant under non-saltstress growth conditions.

The term "architecture" as used herein encompasses the appearance or morphology of a plant, including any one or more structural features or combination of structural features thereof. Such structural features include the shape, size, number, position, texture, arrangement, and pattern of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, leaf, shoot, stem, petiole, trichome, flower, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, cambium, wood, heartwood, parenchyma, aerenchyma, sieve element, phloem or vascular tissue, amongst others. Modified architecture therefore includes all aspects of modified growth of the plant.

The present invention relates to methods to alter growth characteristics of a plant or methods to produce plants with altered growth characteristics, wherein the growth characteristics comprise any one or more selected from: increased yield, increased biomass, increased total above ground area, increased plant height, increased number of tillers, increased number of first panicles, increased number of second panicles, increased number of filled seeds, increased total seed yield per plant, increased harvest index, increased thousand kernel weight, increased Tmid, increased T45 or t90, increased A42 or an altered growth curve. Also the present invention provides methods to alter one of the above mentioned growth characteristics, without causing a penalty on one of the other growth characteristics, for example increased the above ground green tissue area while retaining the same number of filled seeds and the same seed yield.

The invention also relates to use of a nucleic acid sequence capable of modulating expression of a nucleic acid encoding a NHX protein and/or capable of modulating the activity and/or levels of a NHX protein to alter plant growth characteristics wherein said plants are grown

under non-saltstressed growing conditions. Also the invention relates to use of a protein capable of modulating expression of a nucleic acid encoding a NHX protein and/or capable of modulating the activity and/or levels of a NHX protein to alter plant growth characteristics of plant wherein said plants are grown under non-saltstressed growing conditions. Preferably said growth characteristics are improved and encompass for example increased yield/biomass, modified architecture, increased seed yield and increased harvest index. Preferably, the invention encompasses the use of a nucleic acid sequence as mentioned above, wherein said nucleic acid encodes a NHX protein. Further the nucleic acid is preferably encoding an NHX1 protein or is further preferably essential similar to SEQ ID NO 1, 3, 5 or 7 or 11, or a portion thereof or a nucleic acid capable of hybridizing therewith. Further preferably, the nucleic acid is in the sense orientation and/or is controlled by a seed-speciffic promoter such as the prolamin promoter. Preferably, the invention encompasses the use of a protein as mentioned above, wherein said protein is a NHX protein. Further said protein is preferably an NHX1 protein or a protein essentially similar to SEQ ID NO 2, 4, 6 or 8 or a homologue derivative or functional fragment thereof. .

The present invention also relates to the use of a nucleic acid encoding a NHX protein or a homologue, derivative or functional fragment thereof, or to the use of a NHX protein as presented in SEQ ID NO 2 or a homologue, derivative or functional fragment thereof, as a growth regulator. The nucleic acid sequences encoding a NHX protein as hereinbefore described, and the amino acid sequences as hereinbefore described, are useful in modifying the growth characteristics of plants as hereinbefore described. The sequences would therefore find use as growth regulators, such as herbicides or growth stimulators. The present invention also provides a composition comprising a NHX protein represented by any of the aforementioned amino acid sequences or homologues, derivatives or active fragments thereof, for the use as a growth regulator.

Conversely, the sequences according to the present invention may also be interesting targets for agrochemical compounds such as growth regulators (e.g. herbicides or growth stimulators). Accordingly, the present invention encompasses the use of the aforementioned nucleic acid sequences (or a portion of the same or sequences capable of hybridizing with the same) or an amino acid sequences as hereinbefore described (or homologues, derivatives or active fragments thereof), as targets for an agrochemical compound, such as a herbicide or a growth regulator.

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any or more of said steps or features.

The present invention will now be described with reference to the following figures in which:

Fig. 1 is a map of the plasmid p4946 containing the *Oryza sativa* sequence CDS1608 which is the internal code for the cDNA essentially similar to SEQ ID NO 1 and encoding a NHX protein, under control of the rice prolamin promoter (internal code PRO0090), the double terminator sequence T-zein and T-rbcS-deltaGA, located within the borders (the left border (LB repeat, LB Ti C58) and the right border (RB repeat, RB Ti C58)) of the nopaline Ti plasmid; a screenable marker and a selectable marker both cloned within the T-borders and each under a constitutive promoter and each followed by a terminator sequence tNOS and/or a poly A tail; origin of replication (pBR322 (ori + bom)) and a bacterial selectable marker (Sm/SpR).

Fig. 2 presents of all the SEQ ID NO's used in the description of the present invention

Examples

The present invention will now be described with reference to the following examples, which are by way of illustration alone.

DNA Manipulation

Unless otherwise stated, recombinant DNA techniques are performed according to standard protocols described in (Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York) or in Volumes 1 and 2 of Ausubel *et al.* (1994), Current Protocols in Molecular Biology, Current Protocols. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

Example 1: Cloning the Rice NHX gene (CDS1608, SEQ ID NO 1)

The rice gene encoding NHX1 (CDS1608) was amplified by PCR using as template an *Oryza sativa japonica* cv Nipponbare seedling cDNA library (Invitrogen, Paisley, UK). After reverse transcription of RNA extracted from seedlings, the cDNAs were cloned into pCMV Sport 6.0.

Average insert size of the bank was 1.5 kb, and original number of clones was of 1.59×10^7 cfu. Original titer was determined to be 9.6×10^5 cfu/ml, after first amplification of 6×10^{11} cfu/ml. After plasmid extraction, 200 ng of template was used in a 50 µl PCR mix. Primers prm3122 (sense, AttB1 site in italic: 5'-*GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAC*AATGGGGATGGAGGTGG-3') (SEQ ID NO 9) and prm3123 (reverse, complementary, AttB2 site in italic: 5' *GGGGACCACTTTGTACAAGAAAGCTGGG*TGCACTGTTCATCTTCCTCC-3') (SEQ ID NO 10), which include the AttB sites for Gateway recombination, were used for PCR amplification. PCR was performed using Hifi Taq DNA polymerase in standard conditions. The amplified PCR fragment of 1608bp was amplified and purified also using standard methods. The first step of the Gateway procedure, the BP reaction, was then performed, during which the PCR fragment recombines in vivo with the pDONR201 plasmid to produce, according to the Gateway terminology, an "entry clone", p4427. Plasmid pDONR201 was purchased from Invitrogen, as part of the Gateway® technology.

The entry clone p4427 was subsequently used in an LR reaction with p0830, a destination vector used for *Oryza sativa* transformation. This vector contains as functional elements within the T-DNA borders: a plant selectable marker expression cassette; a screenable marker expression cassette; a Gateway cassette intended for LR *in vivo* recombination with the sequence of interest already cloned in the entry clone. A rice GOS2 promoter (PRO0090) for expression of the NHX gene is located upstream of this Gateway cassette.
After the LR recombination step, the resulting expression vector p4946 (Figure 1) can be transformed into the *Agrobacterium* strain LBA4044 and subsequently to *Oryza sativa* plants.

Example 2: Transformation into rice

Calli preparation

Mature dry seeds of the *Oryza sativa* japonica cultivar Nipponbare were dehusked. Sterilization was done by incubating for one minute in 70% ethanol, followed by 30 minutes in 0.2% HgCl2, followed by a 6 times 15 minutes wash with sterile distilled water. The sterile seeds were then germinated on a medium containing 2,4-D (callus induction medium). After incubation in the dark for four weeks, embryogenic, scutellum-derived calli were excised and propagated on the same medium. After two weeks, the calli were multiplied or propagated by subculture on the same medium for another 2 weeks. Embryogenic callus pieces were sub-cultured on fresh medium 3 days before co-cultivation (boost of cell division activity).

Agrobacterium preparation

Agrobacterium tumefaciens strain LBA4044 containing the binary vector p4946 was inoculated on AB medium with the appropriate antibiotics and cultured for 3 days at 28°C. The bacteria were then collected and suspended in liquid co-cultivation medium to a density (OD_{600}) of about 1. The suspension was then transferred to a Petri dish and the calli immersed in the suspension for 15 minutes. The calli tissues were then blotted dry on a filter paper and transferred to solidified co-cultivation medium and incubated for 3 days in the dark at 25°C.

Calli transformation and plant regeneration

Co-cultivated calli were grown on 2,4-D-containing medium for 4 weeks in the dark at 28°C in the presence of a suitable concentration of the selective agent. During this period, rapidly growing resistant callus islands develop. After transfer of this material to a regeneration medium and incubation in the light, the embryogenic potential was released and shoots developed in the next four to five weeks. Shoots were excised from the callus and incubated for 2 to 3 weeks on an auxin-containing medium from which they were transferred to soil. Hardened shoots were grown under high humidity and short days in a greenhouse. Seeds were then harvested three to five months after transplanting. The method yielded single locus transformants at a rate of over 50 % (Aldemita and Hodges, Planta, 199 612-617, 1996; Chan *et al.*, Plant Mol. Biol. 22 (3) 491-506, 1993, Hiei *et al.*, Plant J., 6 (2) 271-282, 1994).

Example 3: Evaluation of transgenic rice transformed with PRO0090-CDS1608 (promoter prolamin – NHX)

Approximately 15 to 20 independent T0 rice transformants were generated. The primary transformants were transferred from tissue culture chambers to a greenhouse for growing and harvest of T1 seed. 5 events, of which the T1 progeny segregated 3:1 for presence/absence of the transgene, were retained. For each of these events, approximately 10 T1 seedlings containing the transgene (hetero- and homo-zygotes), and approximately 10 T1 seedlings lacking the transgene (nullizygotes), were selected by monitoring expression of the screenable marker.

Vegetative growth measurements

The selected T1 plants (approximately 10 with the transgene and approximately 10 without the transgene) were transferred to a greenhouse. Each plant received a unique barcode label to link unambiguously the phenotyping data to the corresponding plant. The selected T1 plants were grown on soil in 10 cm diameter pots under the following environmental settings: photoperiod= 11.5 h, daylight intensity= 30,000 lux or more, daytime temperature= 28°C or higher, night time temperature= 22°C, relative humidity= 60-70%. Transgenic plants and the

corresponding nullizygotes were grown side-by-side at random positions. From the stage of sowing until the stage of maturity each plant were passed several times through a digital imaging cabinet and imaged. At each time point digital images (2048x1536 pixels, 16 million colors) were taken of each plant from at least 6 different angles. Also, pictures were taken from each of the approximately ten selected transgenic plants with the transgene and also from each of the selected plants not containing the transgene. The parameters described below were derived in an automated way from the all the digital images of all the plants, using image analysis software.

(i) Above ground plant area

Plant above ground area was determined by counting the total number of pixels from aboveground plant parts discriminated from the background. This value was averaged for the pictures taken on the same time point from the different angles and was converted to a physical surface value expressed in square mm by calibration. Experiments show that the aboveground plant area measured this way correlates with the biomass of plant parts above ground.

(ii) Plant height

Plant height was determined by the distance between the horizontal lines going through the upper pot edge and the uppermost pixel corresponding to a plant part above ground. This value was averaged for the pictures taken on the same time point from the different angles and was converted, by calibration, to a physical distance expressed in mm. Experiments showed that plant height measured this way correlate with plant height measured manually with a ruler.

(iii) Number of tillers

The number of primary tillers was manually counted at the harvesting of the plants. The tillers were cut off at 3 cm above the pot rim. They were then counted at the cut surface. Tillers that were together in the same sheet were counted as one tiller.

(iv) Number of primary panicles

The tallest panicle and all the panicles that overlap with the tallest panicles when aligned vertically were counted manually, and considered as primary panicles.

(v) Number of secondary panicles

The number of panicles that remained on the plant after the harvest of the primary panicles was counted and considered as secondary panicles.

(vi) Growth curve

The weekly plant area measurements are modelled to obtain a growth curve for each plant, plotted as the value of plant area (in mm2) over the time (in days). From this growth curve the following parameters can be calculated.

(vii) A42

A42 is the plant area at day 42 after sowing as predicted by the growth curve model.

(viii) Tmid

Tmid is the time that a plant needs to grow and to reach 50% of the maximum plant area. Tmid is predicted from the growth curve model.

(ix) T90

T90 is the time that a plant needs to grow and to reach 90% of the maximum plant area. T90 is predicted from the growth curve model.

Seed-related parameter measurements

The mature primary panicles were harvested, bagged, barcode-labelled and then dried for three days in the oven at 37°C. The panicles were then threshed and all the seeds collected. The filled husks were separated from the empty ones using an air-blowing device. After separation, both seed lots were then counted. The empty husks were discarded. The filled husks were weighed on an analytical balance. This procedure resulted in the set of seed-related parameters described below.

(i) Total seed number per plant

The total seed number was measured by counting the number of husks harvested from a plant.

(ii) Number of filled seeds: was determined by counting the number of filled husks that remained after the separation step with the air-blowing device.

(ii) Total seed yield per plant

The total seed yield was measured by weighing all filled husks harvested from a plant.

(iii) Harvest index of plants

The harvest index in the present invention is defined as the ratio between the total seed yield and the above ground area (mm^2), multiplied by a factor 10^6.

(iv) Thousand Kernel Weight (TKW) of plants
This parameter is extrapolated from the number of filled seeds counted, and their total weight.

Statistical analysis: t-test and F-test

A two factor ANOVA (analysis of variants) was used as statistical model for the overall evaluation of plant phenotypic characteristics. An F-test was carried out on all the parameters measured of all the plants of all the events transformed with the gene of the present invention. The F-test is carried out to check for an effect of the gene over all the transformation events and to verify for an overall effect of the gene, also named herein "global gene effect". If the value of the F-test shows that the data are significant, than it is concluded that there is a "gene" effect, meaning that not only presence or the position of the gene is causing the differences in phenotype. The threshold for significance for a true global gene effect is set at 5% probability level for the F-test.

To check for an effect of the genes within an event, i.e., for a line-specific effect, a t-test was performed within each event using data sets from the transgenic plants and the corresponding null plants. "Null plants" or "Null segregants" are the plants treated in the same way as the transgenic plant, but from which the transgene has segregated. Null plants can also be described as the homozygous negative transformants. The threshold for significance for the t-test is set at 10% probability level. Within one population of 4 transformation events, some events can be under or above this t-test threshold. This is based on the hypothesis that a gene might only have an effect in certain positions in the genome, and that the occurrence of this position-dependent effect is not uncommon. This kind of gene effect is also named herein a "line effect of the gene".
The p-value is obtained by comparing the t-value to the t-distribution or alternatively, by comparing the F-value to the F-distribution. The p value then stands for the probability that the null hypothesis (null hypothesis being "there is no effect of the transgene") is correct.

Example 4: Results of the evaluation of transgenic plants transformed with PRO0090-CDS1608 (promoter GOS2- NHX)

Vegetative growth and seed yield was measured according to the methods as described above. It was found that the thaosand kernel weight was increased in the rice plants stably transformed with a nucleic acid comprising a NHX gene (a gene essentially similar to SEQ ID NO 3 (CDS1608)) and that the transgenic plants have an increased harvest index and therefore an increase in yield, compared to the nullizygotes without the NHX transgene.

Harvest index

The values for the harvest of the plants are summarized in Table 2. The results from the F test show that there is an overall gene effect of increase in harvest index in the population of transgenic plants comprising the NHX transgene, compared with the nullizygote population. The general increase in harvest index is 19%, with the probability of the populations being equal of 0.0428. For one line (line 119728), the increase in harvest index was as high as 32% with a probability value of 0.0357.

Table 2: Harvest index

Each row corresponds to one event, for which the harvest index has been determined for the transgenic plants (TR) and the null plants (null). The numeric difference between the positive plants, having the transgene and the negative plants, without the transgene is given (dif) as well as the percentage of difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row presents the average numbers for all events. In the last row, the p-value stands for the p-value derived from the F-test.

harvestIndex					
Line	TR	null	dif	% dif	p-value
119728	86.5	65.7	20.71	32	0.0357
119731	65	53.5	11.54	22	0.2586
119763	64.9	53.8	11.18	21	0.2729
119766	22.2	29.6	-7.38	-25	0.508
119769	32.5	25.4	7.11	28	0.4844
Overall	**55.6**	**46.6**	**9.07**	**19**	**0.0428**

Thousand kernel weight

The values for the thousand kernel weight are summarized in Table 3. The results from the F test show that there is an overall gene effect of increase in thousand kernel weight in the population of transgenic plants comprising the NHX transgene, compared with the nullizygote population. The general increase in thousand kernel weight is 4%, with the probability of the populations being equal of 0.0337. For the plant line 119728, previously identified for increase in harvest index, it has been demonstrated that the thousand kernel weight is also increased with 11%, with a probability value of 0.0088. Also for another line, line 119767, an increase in thousand kernel weigh was demonstrated, reaching values of 15% increase, when compared to the nullizygotes. For this line the probability value of the increase in thousand kernel weigh is 0.003.

Table 3: Thousand kernel weight

Each row corresponds to one event, for which the thousand kernel weight has been determined for the transgenic plants (TR) and the null plants (null). The numeric difference between the positive plants, having the transgene and the negative plants, without the transgene is given (dif) as well as the percentage of difference between these plants (% dif). P-value stands for the probability produced by the t-test for each plant line. The last row presents the average numbers for all events. In the last row, the p-value stands for the p-value derived from the F-test.

TKW					
Line	TR	null	dif	% dif	p-value
119728	22.9	20.7	2.18	11	0.0088
119731	21.6	22.1	-0.51	-2	0.5479
119763	22.5	21.3	1.18	6	0.1665
119766	19.1	21	-1.91	-9	0.0427
119769	20.5	17.9	2.6	15	0.003
Overall	**21.4**	**20.6**	**0.8**	**4**	**0.0337**

The transformed plants of line 119728 further showed also increase plant height with a value of 9% increase in height, compared tocorresponding nullizygotes and with a probability value of 0.0526. This parameter typically indicated that the methods of the resent invention are capable of improvinf plant growth, plant green biomass production, plant yield and plant architecture.

Further, the transformed plant of this same plant line 119728 also show an increase in seed yield compared to the wild-type plants. The weight of total amount of seeds was 36% higher in these transformed plants with the NHX1 gene, than the corresponding nullizygotes, with a probability value of 0.0509.

From these evaluation data it is clear that there was a variation between the different transformation events (different plant events each transformed with the CDS1608 NHX gene). It is well known to persons skilled in the art, that the expression of transgenes in plants, and hence also the phenotypical effect due to expression of such transgene, can differ dramatically among different independently obtained transgenic lines and progeny thereof. The transgene present in different independently obtained transgenic plants differ from each other by the chromosomal insertion locus as well as by the number of transgene copies inserted in that locus and the configuration of those transgene copies in that locus. Differences in expression

levels can be ascribed to influence from the chromosomal context of the transgene (the so-called position effect) or from silencing mechanisms triggered by certain transgene configurations (e.g. inwards facing tandem insertions of transgenes are prone to silencing at the transcriptional or post-transcriptional level).

The exact configuration and insertion loci of the different events have not yet been determined, and expression levels have not been measured. But differences in these will clearly have an impact on the phenotypic parameters that have been measured. In some cases, negative effects may be observed for example when an essential gene is totally silenced instead of being overexpressed (or misexpressed).

Example 5: Zea Mays plants, comprising a NHX transgene, show improved growth characteristics

The vector as presented in figure 1 or a similar vector comprising a similar expression cassette as SEQ ID NO 10, is suitable for transformation into a *Zea Mays* plant, following the agrobacterium-mediated transformation protocol described previously in Frame et al. (*Agrobacterium tumefaciens-mediated transformation of maize embryos using a standard binary vector system*. Plant Physiol. 2002 May;129(1):13-22). The plants are grown under normal growth conditions, meaning no salt-stress or other stress conditions. The plants are evaluated by checking all the growth parameters as described above in example 3. The transformed corn plants show improved growth characteristics on the level of green biomass and seeds. In particular the seed size, seed weight, total seed yield, total number of filled seeds, thousand kernel weigh and the harvest index are increased in the transformed plants compared to the corresponding nullizygotes.

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Claims

1. Method for modifying plant growth characteristics, comprising modulating expression in a plant of a nucleic acid sequence encoding a NHX protein and/or modulating activity and/or levels in a plant of a NHX protein, wherein said plant is grown under non-saltstress conditions.

2. Method for modifying plant growth characteristics, comprising exogenous application of one or more compounds or elements capable of modulating expression of a gene encoding a NHX protein and/or capable of modulating activity and/or levels of a NHX protein.

3. Method according to claim 1 or 2, wherein said modulation is effected, by recombinant means or by chemical means.

4. Method according to any of claims 1 to 3, wherein said modulating expression comprises introducing into a plant a nucleic acid sequence encoding a NHX protein or a homologue, derivative or active fragment thereof.

5. Method for the production of a plant, having modified growth characteristics when grown under non-saltstress conditions, comprising introduction, into a plant, a nucleic acid sequence capable of modulating expression of a gene encoding a NHX protein and/or capable of modulating activity and/or levels of a NHX protein.

6. Method according to any of claim 1 to 5, which method comprises:
   a) Introducing into a plant or a plant cell a nucleic acid sequence encoding a NHX protein or a homologue or a fragment thereof;
   b) Cultivating said plant cell under conditions promoting regeneration and mature plant growth.

7. Method according to any of claims 1 to 6, wherein said growth characteristic is improved, preferably wherein said growth characteristic is any one or more of the characteristics chosen from the group consisting of increased yield/biomass and/or modified plant architecture, such as for example increased above ground area, increased number of first panicles, increased plant height, increased total number of seeds, increased number of filled seeds, increased total seed weight, increased harvest index and increased thousand kernel weight, each relative to the corresponding wild plant and when said plant is grown under non-saltstress conditions.

8. Method according to any of claim 1 to 7, wherein said nucleic acid sequence encoding a NHX protein is in the sense orientation and is under the control of a tissue specific promoter, preferably a seed-specific promoter, further preferably an endosperm-specific promoter such as for example a prolamin promoter.

9. Method according to any of claim 1 to 8, wherein said nucleic acid sequence encodes an NHX1 protein, further preferred, said nucleic acid is derived from a monocotyledoneous plant, for example from the family Poaceae, further preferably from *Oryzaceae*, most preferably is a nucleic acid as presented in SEQ ID NO 1, 3, 5 or 7 or a portion or fragment thereof, or a sequence capable of hybridizing therewith or a sequence having at least, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% sequence identity therewith or a nucleic acid encoding a protein as presented in SEQ ID NO 2, 4, 6 or 8 or a homologue, a derivative or a functional fragment thereof or wherein said NHX gene is part of an expression cassette comprising a seed-specific promoter, for example an expression cassette as presented by SEQ ID NO 11.

10. Method for the selection of pants having altered growth characteristics under non-saltress growing conditions, which altered growth characteristics are based on altered activity of the NHX gene.

11. An isolated nucleic acid, encoding NHX like protein or a fragment thereof, selected from the group consisting of
    a. a nucleic acid sequence encoding an amino acid sequence as presented in SEQ ID NO 4 or 6 or 8;
    b. a nucleic acid sequence depicted in SEQ ID NO 3 or 5 or 7 or 11, or the complementary strand thereof;
    c. nucleic acid sequences which are degenerated as a result of the genetic code to the nucleic acid sequences defined in (a) or (b);
    d. nucleic acid sequences which are allelic variants to the nucleic acid sequences defined in (a) or (b);
    e. nucleic acid sequences which hybridise, preferably under stringent conditions, to sequences defined in (a), (b), (c) or (d);
    f. nucleic acid sequences which have 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to any one or more of the sequence defined in (a), (b), (c) or (d)

g. nucleic acid sequences encoding a protein which has 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to any one or more from the sequences defined in SEQ ID NO 4, 6 or 8;

h. nucleic acid sequences encoding a fragment of a protein encoded by a nucleic acid of any one of (a) to (g).

12. An isolated NHX protein comprising at least part of one of the polypeptides selected from the group consisting of:

a) a polypeptide as given in SEQ ID NO 4, 6 or 8,

b) a polypeptide with an amino acid sequence which has at least 50% sequence identity, preferably 60%, 70% sequence identity, more preferable 80% or 90% sequence identity, most preferable 95%, 96%, 97%, 98% or 99% sequence identity to any one or more of the amino acid sequence as given in SEQ ID NO 4, 6 or 8,

c) a polypeptide encoded by a nucleic acid as mentioned above or a homologue, a derivative, an immunologically active and/or functional fragment of a protein as defined in any of (a) to (d).

13. Genetic construct comprising:

a) a nucleic acid sequence capable of modulating expression of a nucleic acid encoding a NHX protein and/or capable of modulating the activity and/or levels of a NHX protein;

b) a control sequence capable of regulating expression of the nucleic acid sequence of (a); and optionally

c) a transcription termination sequence.

14. Genetic construct according to claim 13, wherein said control sequence of (b), comprises a seed-specific promoter, preferably an endosperm-specific promoter.

15. Genetic construct according to claim 13 or 14, wherein said nucleic acid sequence of (a) is a nucleic acid sequence encoding a NHX protein, or a homologue or a derivative or a functional fragment thereof, preferably a nucleic acid as presented in SEQ ID NO 1, 3, 5 or 7 or a portion or fragment thereof, or a sequence capable of hybridizing therewith or a sequence having at least, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99% sequence identity therewith and/or wherein said nucleic acid is a nucleic acid according to claim 6.

16. Host cell having altered, preferably improved, growth characteristics when grown under non-saltstress conditions, said host cell containing an isolated nucleic acid molecule encoding a NHX protein and/or containing a nucleic acid according to claim 11 and/or containing a genetic construct according to any of claim 13 to 14.

17. Transgenic plant having modified, preferably improved growth characteristics when grown under non-saltstress conditions, said plant containing an isolated nucleic acid molecule encoding a NHX protein and/or containing a nucleic acid according to claim 11 and/or containing a genetic construct according to any of claim 13 to 14.

18. Transgenic plant having modified, preferably improved growth characteristics when grown under non-saltstress conditions, characterized in that said plant has modulated expression of a nucleic acid sequence encoding a NHX protein and/or modulated activity and or level of a NHX a protein.

19. Plant obtainable by a method according to any of claims 1 to 10, which plants have modified, preferably improved growth characteristics.

20. Plant part, preferably a harvestable plant part, a propagule or progeny from a plant according to claim 17 or 19.

21. Use of a nucleic acid sequence capable of modulating expression of a nucleic acid encoding a NHX protein and/or capable of modulating the activity and/or levels of a NHX protein to alter plant growth characteristics of a plant, preferably increasing yield/biomass and/or architecture, when said plant is grown under non-saltstress conditions.

22. Use of a nucleic acid sequence according to claim 20, wherein said nucleic acid encodes a NHX protein, preferably an NHX1 protein .

23. Use of a protein capable of modulating expression of a nucleic acid encoding a NHX protein and/or capable of modulating the activity and/or levels of a NHX protein to alter plant growth characteristics of a plant, preferably increasing yield/biomass and/or architecture, wherein said plant is grown under non-saltstress conditions.

24. Use of a protein according to claim 23, wherein said protein is a NHX protein, preferably an NHX1 protein.

25. Use of a nucleic acid sequence in breeding programs, which nucleic acid sequence is capable of modulating expression of a nucleic acid encoding a NHX protein or capable of modulating the activity and/or levels of a NHX protein.

26. Use of a gene encoding a NHX protein or a protein having NHX activity as a growth regulator, such as a herbicide or a growth stimulator.

27. Use of a gene encoding a NHX protein or a NHX protein as a target of an agrochemical compound, such as a herbicide or a growth stimulator.

28. Composition comprising a NHX protein, for use as a growth regulator.

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Abstract

Plants having modified growth characteristics and a method for making the same

The present invention concerns a method for modifying the growth characteristics of a plant by modulating expression in a plant of a nucleic acid sequence encoding a NHX protein and/or modulating activity in a plant of a NHX protein. NHX proteins are Na+/H+ antiporter proteins involved in the response of plants to saltstress. Now it has now been shown for the first time that NHX transfomed plants, grown under normal growth conditions, under non-saltstress conditions, show improved growth characteristics compared to the wild-type plants also grown under the same normal growth conditions. Further the present invention concerns nucleic acid sequences encoding a NHX protein. The invention also relates to transgenic plants, grown in normal growth conditions and having modified growth characteristics compared to the wild-type plants, which plants have modulated expression of a nucleic acid encoding a NHX protein. More in particular a NHX1 encoding gene from *Oryza sativa* was introduced in the sense orientation and under the control of a seed-specific promoter into a rice plant, resulting in the modulation of various growth characteristics, such as improved biomass production and improved seed yield.

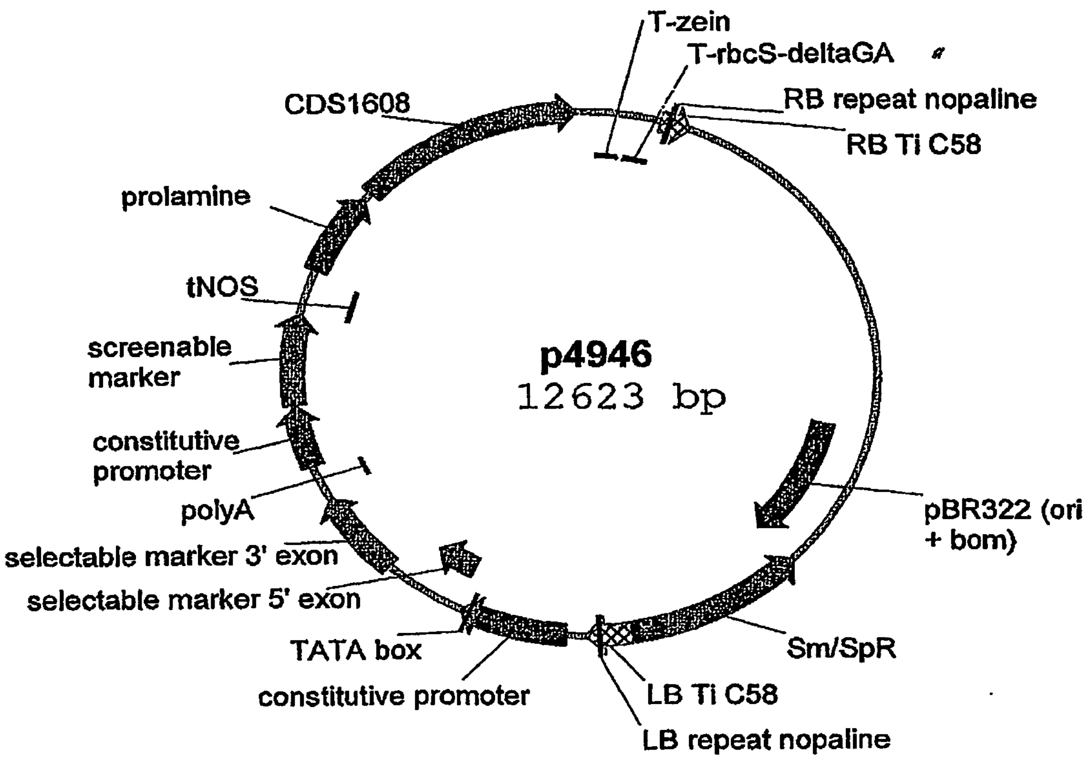
T-zein
T-rbcS-deltaGA
RB repeat nopaline
RB Ti C58
CDS1608
prolamine
tNOS
screenable marker
constitutive promoter
polyA
selectable marker 3' exon
selectable marker 5' exon
TATA box
constitutive promoter
p4946
12623 bp
pBR322 (ori + bom)
Sm/SpR
LB Ti C58
LB repeat nopaline

FIGURE 1

SEQ ID NO 1: coding sequence for *Oryza sativa* NHX1 protein

GAGAAGAGAGTTTTGTAGCGAGCTCGCGCGAATGCGAAGCCAACCGAGAGAGGTCTCGATAC
CAAATCCCGATTTCTCAACCTGAATCCCCCCCCCACGTTCCTCGTTTCAATCTGTTCGTCTG
CGAATCGAATTCTTTGTTTTTTTTTCTCTAATTTTACCGGGAATTCTCGAATTAGGCATTCA
CCAACGAGCAAGAGGGGAGTGGATTGGTTGGTTAAAGCTCCGCATCTTGCGGCGGAAATCTC
GCTCTCTTCTCTGCGGTGGGTGGCCGGAGAAGTCGCCGCCGGTGAGGC***ATG***GGGATGGAGGT
GGCGGCGGCGCGGCTGGGGGCTCTGTACACGACCTCCGACTACGCGTCGGTGGTGTCCATCA
ACCTGTTCGTCGCGCTGCTCTGCGCCTGCATCGTCCTCGGCCACCTCCTCGAGGAGAATCGC
TGGGTCAATGAGTCCATCACCGCGCTCATCATCGGGCTCTGCACCGGCGTGGTGATCTTGCT
GATGACCAAAGGGAAGAGCTCGCACTTATTCGTCTTCAGTGAGGATCTCTTCTTCATCTACC
TCCTCCCTCCGATCATCTTCAATGCAGGTTTTCAGGTAAAGAAAAAGCAATTGTTCCGGAAT
TTCATGACGATCACATTATTTGGAGCCGTCGGGACAATGATATCCTTTTTCACAATATCTAT
TGCTGCCATTGCAATATTCAGCAGAATGAACATTGGAACGCTGGATGTAGGAGATTTTCTTG
CAATTGGAGCCATCTTTTCTGCGACAGATTCTGTCTGCACATTGCAGGTCCTCAATCAGGAT
GAGACACCCTTTTTGTACAGTCTGGTATTCGGTGAAGGTGTTGTGAACGATGCTACATCAAT
TGTGCTTTTCAACGCACTACAGAACTTTGATCTTGTCCACATAGATGCGGCTGTCGTTCTGA
AATTCTTGGGGAACTTCTTTTATTTATTTTTGTCGAGCACCTTCCTTGGAGTATTTGCTGGA
TTGCTCAGTGCATACATAATCAAGAAGCTATACATTGGAAGGCATTCTACTGACCGTGAGGT
TGCCCTTATGATGCTCATGGCTTACCTTTCATATATGCTGGCTGAGTTGCTAGATTTGAGCG
GCATTCTCACCGTATTCTTCTGTGGTATTGTAATGTCACATTACACTTGGCATAACGTCACA
GAGAGTTCAAGAGTTACAACAAAGCACGCATTTGCAACTCTGTCCTTCATTGCTGAGACTTT
TCTCTTCCTGTATGTTGGGATGGATGCATTGGATATTGAAAAATGGGAGTTTGCCAGTGACA
GACCTGGCAAATCCATTGGGATAAGCTCAATTTTGCTAGGATTGGTTCTGATTGGAAGAGCT
GCTTTTGTATTCCCGCTGTCGTTCTTGTCGAACCTAACAAAGAAGGCACCGAATGAAAAAAT
AACCTGGAGACAGCAAGTTGTAATATGGTGGGCTGGGCTGATGAGAGGAGCTGTGTCGATTG
CTCTTGCTTACAATAAGTTTACAAGATCTGGCCATACTCAGCTGCACGGCAATGCAATAATG
ATCACCAGCACCATCACTGTCGTTCTTTTTAGCACTATGGTATTTGGGATGATGACAAAGCC
ATTGATCAGGCTGCTGCTACCGGCCTCAGGCCATCCTGTCACCTCTGAGCCTTCATCACCAA
AGTCCCTGCATTCTCCTCTCCTGACAAGCATGCAAGGTTCTGACCTCGAGAGTACAACCAAC
ATTGTGAGGCCTTCCAGCCTCCGGATGCTCCTCACCAAGCCGACCCACACTGTCCACTACTA
CTGGCGCAAGTTCGACGACGCGCTGATGCGACCGATGTTTGGCGGGCGCGGGTTCGTGCCCT
TCTCCCCTGGATCACCAACCGAGCAGAGCCATGGAGGAAGA***TGA***ACAGTGCAAAGAAATGAG
AATGGAATGGTTGATGAGGAGAATACATGTAAAATGTGACAGCAAAAGAGAGAAGGCAAGTT
TTGGGTTTGTAGAGTTTGGCTGCTGCTAATGAGTTGTTGATAGTGCCTATATTCTTCAGAAC
TTCAGATGGTGCCTCACCAAGGCCTAAGAGCCAGGAGGACCTTCTGATAATGGTTCGGGATG
ATTGGTTTGTTCTGTCAGGATGAACCCTAGTGAGTGACACAGGGTGATGTGCTCCGACAACC
TGTAAATTTTGTAGATTAACAGCCCCATTTGTACCTGTCTACCATCTTTAGTTGGCGGGTGT
TCTTTCCTAGTTGCCACCCTGCATGTAAAATGAAATTCTCCGCCAAAATAGATTTGTGTGTA
TAATAATTTTGCTTGGTTG

FIGURE 2

SEQ ID NO 2: ***Oryza sativa*** **NHX1 protein**

MGMEVAAARLGALYTTSDYASVVSINLFVALLCACIVLGHLLEENRWVNESITALIIGLCTG
VVILLMTKGKSSHLFVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFMTITLFGAVGTMISF
FTISIAAIAIFSRMNIGTLDVGDFLAIGAIFSATDSVCTLQVLNQDETPFLYSLVFGEGVVN
DATSIVLFNALQNFDLVHIDAAVVLKFLGNFFYLFLSSTFLGVFAGLLSAYIIKKLYIGRHS
TDREVALMMLMAYLSYMLAELLDLSGILTVFFCGIVMSHYTWHNVTESSRVTTKHAFATLSF
IAETFLFLYVGMDALDIEKWEFASDRPGKSIGISSILLGLVLIGRAAFVFPLSFLSNLTKKA
PNEKITWRQQVVIWWAGLMRGAVSIALAYNKFTRSGHTQLHGNAIMITSTITVVLFSTMVFG
MMTKPLIRLLLPASGHPVTSEPSSPKSLHSPLLTSMQGSDLESTTNIVRPSSLRMLLTKPTH
TVHYYWRKFDDALMRPMFGGRGFVPFSPGSPTEQSHGGR

SEQ ID NO 3: SCRLFL4028D02.g coding sequence for ***Saccharum officinarum*** **NHX partial protein**

tgcctgccggggcccggattggaggcggaccggcggcatggggctcggcgtggtgtcggagc
tggtgcggctgggcgtgctgagcttgacctccgaccacgcgtcggtggtgtccatcaacctg
ttcgtcgcgctgctctgcgcctgcatcgtcctcggccacctcctggaggagaaccgctgggt
caacgagtccatcaccgcgctcatcatcgggctgtgcactggcgtggtgatcctgctgacca
ccaaggggaagagctcgcacatcctcgtcttcagcgaggacctcttcttcatctacctcctc
cctccaatcatcttcaatgccgggttccaggtgaagaagaaacaattcttccggaatttcat
gacaatcacattatttggtgctgttgggacaatgatatccttcttcacaatctctctcggtg
caatagcgatattcagcagaatgaacattggaacgttagatgtcggggatttctcgctatt
ggagctatcttttctgcaacagattctgtctgcacactgcaggtcctccatcaggatgagac
gcccctttgtacagtcttgtgtttggtgaaggagttgtgaacgatgccacgtctgttgtgc
tcttcaacgcactccagaattttgatcttaaccacatcgatgtagccgttgtgctgaagttc
ttgggaaatttctgttatttattcttgtcaagcaccttacttggagtgtttactggattgct
cagtgcctacataattaagaagttatatataggaaggcattccactgaccgtgaggttgcgc
ttatgatgctcatggcttacctctcatatatgttggctgagttgctagatctgagtggtatt
cttactgtattcttctgcggtattgtgatgtcgcattacacttggcataatgtgacagagag
ctcaagagttacaaccaagcacgcctttgcaactttgtccttcattgctgagacttttctct
tcctatatgttgggatggatgccctcgatatcgagaagtgggaatttgccagtgacagcccg
ggcaaatccattggcataagctcgattttgctaggattggttctggtggggagagctgcatt
tgttttcccattgtcgttttgtccaacttgacaaagaagtctccactggagaaataacat
tgagacaacaaattgtaatatggtgggctggactgatgagaggcgccgtgtccattgctctt
gcttacaacaagttcacaagatctggacacactcagctgcacggcaatgcgataatgatcac
cagcacaatcact

FIGURE 2 (continued)

SEQ ID NO 4: SCRLFL4028D02.g protein: *Saccharum officinarum* NHXpartial, likely a deletion in the C-terminal part, Identity with OsNHX1 is Identities = 348/424 (82%)

MGLGVVSELVRLGVLSLTSDHASVVSINLFVALLCACIVLGHLLEENRWVNESITALIIGLC
TGVVILLTTKGKSSHILVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFMTITLFGAVGTMI
SFFTISLGAIAIFSRMNIGTLDVGDFLAIGAIFSATDSVCTLQVLHQDETPLLYSLVFGEGV
VNDATSVVLFNALQNFDLNHIDVAVVLKFLGNFCYLFLSSTLLGVFTGLLSAYIIKKLYIGR
HSTDREVALMMLMAYLSYMLAELLDLSGILTVFFCGIVMSHYTWHNVTESSRVTTKHAFATL
SFIAETFLFLYVGMDALDIEKWEFASDSPGKSIGISSILLGLVLVGRAAFVFPLSFLSNLTK
KSPLEKITLRQQIVIWWAGLMRGAVSIALAYNKFTRSGHTQLHGNAIMITSTILT

SEQ ID NO 5: SCRURT2005c05.g coding sequence for *Saccharum officinarum* NHX partial

CCAGCGCGGGACGACACCACTTCTCTCCTCCCCGGATCACCCACCGAACCCACGCGAAGCCG
GCGTCCAGATCTTCTCCCCCGCGGTTCAGATCCCCGGACCGAGCCGACGCCGCCTGAAGAAT
CTGCAGCAGCGGCGAGGTTGCTTTGAGCTGTGGTGGATCTCCTGGCCGGCGTGGCGACATGG
GGCTGGGTTTGGGAGCTCTTCTCAAATCCGGCGGCCTCTCGGTCTCGGACTACGACGCCATC
GTCTCGATCAACATCTTCATCGCGCTGCTCTGCAGCTGCATTGTCATCGGCCACTTGCTGGA
AGGGAACCGATGGGTCAACGAGTCCATCACCGCGCTTGTCATGGGCCTCATCACGGGAGGCG
TGATCCTGCTGGTCACTAATGGGACAAACTCACGCATTCTTGTGTTCAGCGAGGACCTGTTT
TTCATCTATTTGCTTCCGCCGATAATCTTCAATGCCGGGTTTCAAGTAAAGAAAAAGCAATT
CTTCCGCAACTTTATAACAATTATTTTGTTTGGTGCTGTTGGAACTCTGATTTCCTTTGTAA
TAATCTCTCTTGGTGCTATGGGATTGTTCAAAAAACTTGATGTTGGTCCACTCGAGCTTGGG
GACTATCTTGCAATTGGTGCTATTTTCTCTGCGACAGATTCTGTTTGCACCTTACAGGTGCT
TAACCAGGATGAAACACCCCTACTCTATAGTCTAGTTTTTGGTGAAGGTGTTGTTAATGATG
CCACATCTGTTGTGCTCTTCAATGCAATTGAAAACCTTGATATTGCTAATTTTGATGCTATT
GTTCTGTTGAATTTCGTCGGAAAATTTCTCTACCTGTTCTTCACCAGCACCATACTTGGAGT
AGCTACTGGGTTGCTTAGTGCGTACATTATCAAGAAGCTCTGTTTTGCCAGACATTCAACTG
ATAGAGAAGTTTCTATCATGATACTCATGGCATACCTTTCGTACATGTTGTCAATGCTGTTG
GACCTGAGTGGCATTCTTACTGTCTTCTTCTGTGGAATAGTAATGTCACATTACACTTGGCA
TAATGTGACAGAAAGTTCTAGGGTTACCACTAAGCATACTTTTGCAACTTTATCATTCATCG
CAGAAATTTTCCTCTTCCTCTATGTTGGGATGGATGCATTGGACATTGAGAAGTGGAAATTA
GCTAGTAGCAGTCCTAAGAAACCAATTGCTTTAAGTGCAATTATATTGGGATTGGTTATGGT
TGGAAGAGCGGCATTTGTATTCCCTTTGTCGTTCCTATCCAACCTAAGTAAAAAGGAGGCCC
GTCCAAAGATCTCCTTCAAGCAACAAGTAATCATATGGTGGGCTGGTCTCATGAGAGGAGCA
GTGTCAATTGCACTTGCCTATAACAAGTTTACATCATCTGGTCATACTGAAGTGCGAGTTAA
TGCTATCATGATCACCAGCACAGTTATTGTTGTTCTATTCAGCACAATGGTTTTCGGTCTGC
TGACCAAGCCGCTGCTTAGTCTTCTCATCCCACCAAGGACTGGCTTGAACACATCATCTCTG
CTCTCCAGCCAGTCTATTCTGGACCCACTTCTTACTAGCATGGTGGGGTCTGACTTTGATGT
AGGACAGATCAACTCCCCCCAGTACAACCTCCAGTTTATTCT

FIGURE 2 (continued)

SEQ ID NO 6: SCRURT2005c05.g protein: *Saccharum officinarum* NHX partial

MGLGLGALLKSGGLSVSDYDAIVSINIFIALLCSCIVIGHLLEGNRWVNESITALVMGLITG
GVILLVTNGTNSRILVFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFITIILFGAVGTLISF
VIISLGAMGLFKKLDVGPLELGDYLAIGAIFSATDSVCTLQVLNQDETPLLYSLVFGEGVVN
DATSVVLFNAIENLDIANFDAIVLLNFVGKFLYLFFTSTILGVATGLLSAYIIKKLCFARHS
TDREVSIMILMAYLSYMLSMLLDLSGILTVFFCGIVMSHYTWHNVTESSRVTTKHTFATLSF
IAEIFLFLYVGMDALDIEKWKLASSSPKKPIALSAIILGLVMVGRAAFVFPLSFLSNLSKKE
ARPKISFKQQVIIWWAGLMRGAVSIALAYNKFTSSGHTEVRVNAIMITSTVIVVLFSTMVFG
LLTKPLLSLLIPPRTGLNTSSLLSSQSILDPLLTSMVGSDFDVGQINSPQYNLQFI

SEQ ID NO 7: SCCCLR1066F02.g coding sequence for *Saccharum officinarum* NHX partial

ccagcgcgggacgacaccacttctctcctccccggatcacccaccgaacccacgcgaagccg
gcgtccagatcttctcccccgcggttcagatccccggaccgagccgacgccgcctgaagaat
ctgcagcagcggcgaggttgctttgagctgtggtggatctcctggccggcgtggcgacatgg
ggctgggtttgggagctcttctcaaatccggcggcctctcggtctcggactacgacgccatc
gtctcgatcaacatcttcatcgcgctgctctgcagctgcattgtcatcggccacttgctgga
agggaaccgatgggtcaacgagtccatcaccgcgcttgtcatgggcctcatcacgggaggcg
tgatcctgctggtcactaatgggacaaactcacgcattcttgtgttcagcgaggacctgttt
ttcatctatttgcttccgccgataatcttcaatgccgggtttcaagtaaagaaaaagcaatt
cttccgcaactttataacaattattttgtttggtgctgttggaactctgatttcctttgtaa
taatctctcttggtgctatgggattgttcaaaaaacttgatgttggtccactcgagcttggg
gactatcttgcaattggtgctattttctctgcgacagattctgtttgcaccttacaggtgct
taaccaggatgaaacacccctactctatagtctagtttttggtgaaggtgttgttaatgatg
ccacatctgttgtgctcttcaatgcaattgaaaccttgatattgctaattttgatgctatt
gttctgttgaatttcgtcggaaatttctctacctgttcttcaccagcaccatacttggagt
agctactgggttgcttagtgcgtacattatcaagaagctctgttttgccagacattcaactg
atagagaagtttctatcatgatactcatggcatacctttcgtacatgttgtcaatgctgttg
gacctgagtggcattcttactgtcttcttctgtggaatagtaatgtcacattacacttggca
taatgtgacagaaagttctagggttaccactaagcatacttttgcaactttatcattcatcg
cagaaattttcctcttcctctatgttgggatggatgcattggacattgagaagtggaaatta
gctagtagcagtcctaagaaaccaattgctttaagtgcaattatattgggattggttatggt
tggaagagcggcatttgtattccctttgtcgttcctatccaacctaagtaaaaaggaggccc
gtccaaagatctccttcaagcaacaagtaatcatatggtgggctggtctcatgagaggagca
gtgtcaattgcacttgcctataacaagtttacatcatctggtcatactgaagtgcgagttaa
tgctatcatgatcaccagcacagttattgttgttctattcagcacaatggttttcggtctgc
tgaccaagccgctgcttagtcttctcatcccaccaaggactggcttgaacacatcatctctg
ctctccagccagtctattctggacccacttcttactagcatggtggggtctgactttgatgt
aggacagatcaactccccccagtacaacctccagtttattct

FIGURE 2 (continued)

SEQ ID NO 8: SCCCLR1066F02.g protein: *Saccharum officinarum* NHX partial

MGLALGDPPADYASIASVGLFVALMCVCIIVGHLLEENRWMNESITALFIGLGTGAVILFAS
SGKHSRVLQFSEDLFFIYLLPPIIFNAGFQVKKKQFFRNFITITLFGAVGTLISFTVISLGA
LGLVSRLNIGALELGDYLALGAIFSATDSVCTLQVLSQDETPFLYSLVFGEGVVNDATSVVL
FNAIQNFDLGDISGAKLLNFIGSFLYLFGTSTFLGVASGLLSAYIIKKLYFGRHSTDREVSI
MMLMAYLSYMLAELLDLSGILTVFFCGIVMSHYTWHNVTESSRVTTKHAFATLSFIAETFLF
LYVGMDALDIEKWKIVSQTYSPVKSIALSSTILALVLVSRAAFVFPLSFLSNLTKKTPNGKI
S

SEQ ID NO 9: primer prm3122 Forward

*GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAC*AATGGGGATGGAGGTGG

SEQ ID NO 10: primer prm3123 reverse

*GGGGACCACTTTGTACAAGAAAGCTGGG*TGCACTGTTCATCTTCCTCC

FIGURE 2 (continued)

SEQ ID NO 11: promoter-gene-terminator combination: Sequence of the [PRO0090 (rice prolamin promoter)- CDS1608 - terminator] expression cassette; Promoter prolamine Start: 44 - End: 697; CDS1608 Start: 750 - End: 2357).

agaattcctttcgtcgacccacgtgttgctgaggtatttaaatcttctacatcggcttaggt
gtagcaacacgactttattattattattattattattattattttacaaaatataaaa
tagatcagtccctcaccacaagtagagcaagttggtgagttattgtaaagttctacaaagct
aatttaaaagttattgcattaacttatttcatattacaaacaagagtgtcaatggaacaatg
aaaaccatatgacatactataattttgtttttattattgaaattatataattcaaagagaat
aaatccacatagccgtaaagttctacatgtggtgcattaccaaaatatatatagcttacaaa
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FIGURE 2 (continued)